EINFLUSS DES CHEMOKINS CXCL12 AUF DIE ENTWICKLUNG NEURONALER STRUKTUREN

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Abkürzungsverzeichnis

Abb, Abbildung
cAMP, Zyklisches Adenosinmonophosphat
CP, Kortikalplatte
CR, Cajal-Retzius
DiI, 1,1', di-octadecyl -3,3,3'3'-tetramethylindocarbocyanine perchlorate
E, Embryonaltag
FAK, fokale Adhäsions-Kinase
GABA, γ-Aminobuttersäure
GE, Ganglienhügel
GFP, grün-fluoreszierendes Protein
GnRH, *Gonadotropin-Releasing*-Hormon
GRK, G-Protein-Rezeptor-Kinase
IHC, Immunhistochemie
IPC, *intermediate progenitor cells* (auch basale Vorläuferzellen)
ISH, *in situ* Hybridisierung
IZ, Intermediärzone
LGE, lateraler Ganglienhügel
MAP, *mitogen-activated protein*
MGE, medialer Ganglienhügel
mRNA, *messenger* RNA
MZ, Marginalzone
PGC, primordiale Keimzellen
PKC, Protein-Kinase C
PSPB, *Pallium-Subpallium*-Grenze
RFP, rot-fluoreszierendes Protein
RGC, radiale Gliazellen
RNA, Ribonukleinsäuren
SDF-1 (CXCL12), *stromal cell-derived factor-1*
SVZ, Subventrikularzone
TCA, thalamokortikale Axone
u. a., unter anderem
VNO, Vomeronasalorgan
VZ, Ventrikulärzone
z. B., zum Beispiel

1 Zusammenfassung

Das Chemokin CXCL12 und seine Rezeptoren CXCR4 und CXCR7 beeinflussen zahlreiche physiologische und pathophysiologische Prozesse. Diese kumulative Doktorarbeit vereint drei Publikationen, in denen die Entstehung neuronaler Strukturen unter dem Einfluss dieses Systems beschrieben wird. Es wird gezeigt, dass CXCL12 das Wachstum thalamokortikaler Axone (TCA) sowie die Migration von zerebrokortikalen Interneuronen und *Gonadotropin-Releasing*-Hormon (GnRH)-Neuronen steuert. Während die CXCL12/CXCR4-Interaktion hauptsächlich die Chemotaxis vermittelt, moduliert CXCR7 diesen Prozess, indem er CXCL12 aus dem Gewebe entfernt/wegfängt (englisch *to scavenge*) und dadurch die lokale Verfügbarkeit von CXCL12 reguliert. Der Verlust von CXCR7 führt zu einer Anreicherung von CXCL12, wodurch CXCR4 gesteigert aktiviert, internalisiert und abgebaut wird. Die Abnahme des CXCR4-Proteins führt zu einem Abschwächen/Ausfall der CXCL12/CXCR4-vermittelten chemotaktischen Antwort und dadurch zu zellulären Fehlverteilungen.

Das Manuskript I untersucht den Einfluss von CXCL12 auf das pränatale TCA-Wachstum. In dem Modellorganismus Maus wurde mittels *in situ* Hybridisierung (ISH) und Immunhistochemie (IHC) erstmals CXCR4 in dem sich entwickelnden Thalamus und in TCA nachgewiesen. Die thalamische Expression von *Cxcr4* ist dabei dynamisch reguliert, wobei die frühe Phase der Thalamus-Entwicklung (Embryonaltag (E)12-14) mit hoher und eine späte Phase (ab E15) mit sinkender *Cxcr4*-Expression einhergeht. Dem Thalamus angrenzende Meningen exprimieren CXCL12, welches CXCR4 in den lateralen Thalamuskernen aktiviert. Eine transgene *Cxcr4*-GFP-Reporter-Mauslinie ermöglichte die Visualisierung der TCA und dadurch deren Entwicklung entlang definierter Routen zu verfolgen. Die TCA ziehen vom Thalamus ventral in die *Capsula interna* und erreichen den zerebralen Kortex zwischen E13 und E14. Hier treffen sie auf eine zweite CXCL12-Quelle, die von kortikalen Meningen und Projektionsneuron-Vorläufern (*intermediate progenitor cells*, IPC) in der Subventrikularzone (SVZ) ausgeht. Um die Rolle von CXCL12 bei der Entwicklung der TCA zu untersuchen, wurden *Cxcl12*-defiziente und *Cxcl12*-überexprimierende Mäuse verwendet. Die Axone wurden mittels DiI *tracing* oder durch das Einkreuzen des *Cxcr4*-GFP-Reporters dargestellt. Während der Verlust von CXCL12 zur verkürzten intrakortikalen Ausdehnung der TCA führt, beschleunigt die CXCL12-Überexpression das TCA-Wachstum bis hin zur verfrühten Kortikalplatten (CP)-Invasion. Expressions- und *in vitro*-Analysen deuten auf ein Wechselspiel zwischen dem wachstumsfördernden Effekt von CXCL12/CXCR4 und dem TCA verlangsamenden Einfluss von Slit1/Robo1 hin. Schlussendlich zeigte eine *Tbr2*-Cre-vermittelte Deletion von *Cxcl12* in

der kortikalen SVZ, dass IPC-gebildetes CXCL12 relevant für das intrakortikale TCA-Wachstum und die Verteilung kortikaler Interneurone ist. Damit verknüpft CXCL12 die IPC-basierte Amplifikation kortikaler Projektionsneurone mit ihrer effizienten Versorgung durch zukünftige neuronale Netzwerkpartner (thalamische Eingänge, Interneurone).

Die Manuskripte II und III zeigen eine entscheidende Rolle des CXCL12/CXCR4/CXCR7-Systems bei der neuronalen Migration kortikaler Interneurone und hypothalamischer GnRH-Neurone. Es ist bekannt, dass CXCL12 die Interneurone auf ihren stereotypen Routen in der Marginalzone (MZ) und SVZ hält und migrierende GnRH-Neurone von ihrem Entstehungsort im Vomeronasalorgan durch das nasale Mesenchym zum Vorderhirn leitet. Darauf aufbauend wird hier vergleichend gezeigt, dass entweder *Cxcl12*-, *Cxcr4*- oder *Cxcr7*-Nullmutanten einen ähnlichen Migrationsdefekt hervorbringen: Kortikale Interneurone verlassen ihre stereotypen Routen und akkumulieren verfrüht in der Kortikalplatten/Subplatten (CP/SP)-Region; GnRH-Neurone verbleiben zum Teil in der Nasenregion und erreichen ihr Ziel im Hypothalamus nicht. Dieser Befund wirft die Frage nach der Funktion von CXCR7 bei diesen Migrationsprozessen auf, da er als atypischer Chemokin-Rezeptor keine G-Protein-vermittelte Chemotaxis auslöst. Mit *Cxcr7*$^{-/-}$ Mäusen, welche zusätzlich ein transgenes CXCL12-RFP-Fusionsprotein produzieren, konnte *in vivo* dargelegt werden, dass CXCR7 die effiziente Aufnahme und dadurch die Verteilung von CXCL12 in Geweben moduliert. Bei Verlust dieser CXCR7-vermittelten *scavenger*-Funktion kommt es zur Umverteilung bzw. Anreicherung von CXCL12. Dieses Überangebot führt zu einer Hyperaktivierung von CXCR4, welcher anschließend verstärkt internalisiert und abgebaut wird. Über seine CXCL12-*scavenger*-Funktion beeinflusst CXCR7 also die CXCL12/CXCR4-Interaktion indirekt, indem er die Menge des Liganden steuert und dadurch das CXCR4-Protein vor Hyperaktivierung und Abbau schützt. Dabei kann man zwischen zellautonomer und nicht-zellautonomer CXCR7-Wirkung unterscheiden. Bei der GnRH-Neuron-Migration ist es ein nicht-zellautonomer Prozess, da GnRH-Neurone selbst keinen CXCR7 besitzen und die CXCL12-Menge/Verteilung über mesenchymal-exprimierten CXCR7 reguliert wird. Dahingegen sind kortikale Interneurone eine der wenigen Zellpopulationen, die sowohl CXCR4 als auch CXCR7 besitzen. Zusammen mit vorhergehenden Arbeiten wurde im Manuskript II nachgewiesen, dass die CXCR7-*scavenger*-Funktion in Interneuronen (zellautonom) für den Erhalt der CXCL12/CXCR4-vermittelten Chemotaxis entlang der stereotypen Routen (MZ, SVZ) essentiell ist.

Zusammenfassend belegt diese Arbeit ein komplexes Zusammenspiel von CXCL12 und seiner Rezeptoren bei der Entstehung neuronaler Strukturen.

2 Einleitung

Chemokine sind kleine sezernierte Signalproteine (7-14 kDa) und gehören zu der großen Gruppe der Zytokine (Guyon 2014). Der Begriff Chemokin leitet sich als Kurzform vom **chemo**taktischen Zyto**kin** ab und wurde erstmals im Zusammenhang mit der chemotaktischen Migration von Granulozyten beschrieben (Yoshimura et al. 1987). Evolutionär treten die Chemokine erstmals bei den *Teleostei* (echte Knochenfische) auf und stellen heute die größte Zytokingruppe dar (Bachelerie et al. 2014). Sie werden auf der Grundlage der Position ihrer ersten beiden Zysteinreste in vier Gruppen eingeteilt: CXC, CC, C und CX3C. Eines der bedeutendsten und meist erforschten Chemokine der Klasse CXC ist *stromal cell-derived factor-1* (SDF-1) oder laut aktueller Nomenklatur CXCL12 (C-X-C Motiv und L für Ligand). Die Interaktion zwischen CXCL12 und seinen Sieben-Transmembrandomänen-Rezeptoren CXCR4 und CXCR7 beeinflusst zahlreiche physiologische und pathophysiologische Prozesse. So proliferieren und navigieren zahlreiche Zelltypen im sich entwickelnden und adulten Organismus unter CXCL12-Einfluss (Deverman und Patterson 2009, Guyon 2014, Lewellis und Knaut 2012, Stumm und Hollt 2007). Nach Zellteilung und Migration fangen die Zellen an, sich zu differenzieren und in das Gewebe zu integrieren. Auch an diesen Prozessen ist CXCL12 beteiligt.

Bei Neuronen beispielsweise heißt Integration, Verbindungen mit anderen Neuronen aufzunehmen. Dies geschieht über Neuriten (Nervenzellausläufer), die nach Differenzierungsprozessen in Dendrit und Axon unterschieden werden. Axone können über lange Strecken wachsen und nutzen biochemische Botenstoffe als Wegweiser (Tessier-Lavigne und Goodman 1996). Es konnte gezeigt werden, dass CXCL12 einer dieser Botenstoffe ist, da es axonales Wachstum steigert und Wegfindungsprozesse moduliert (Manuskript I, Stumm und Hollt 2007, Xiang et al. 2002). Nachdem die neuronalen Netzwerke ausgebildet sind, kann CXCL12 in verschiedenen Hirnregionen neuromodulativ wirken. Dabei kommt es nach CXCL12/CXCR4-Interaktion zu einer Modulation spannungsabhängiger Kanäle (Na^+, K^+, Ca^{2+}), zur Aktivierung des GIRK-Kanals (*G protein activated inwardly rectifying* K^+ *channel*) und/oder zu einer Veränderung der Neurotransmitterfreisetzung (z. B. GABA, Glutamat, Dopamin) (Guyon 2014).

Unter pathophysiologischen Bedingungen vermittelt CXCL12 ebenfalls zahlreiche Prozesse, wie beispielsweise Tumorwachstum, Tumormetastasierung und Entzündungsvorgänge. Zusätzlich fungiert der CXCL12-Rezeptor CXCR4 als Ko-Rezeptor bei der Infektion von T-Zellen mit HI-Viren (Guyon 2014, Li und Ransohoff 2008), was neben oben genannten Prozessen CXCL12 zum Gegenstand aktueller medizinischer Forschung macht.

2.1 Die Interaktion von CXCL12 mit seinen Rezeptoren CXCR4 und CXCR7

CXCL12 ist eines der ältesten und konserviertesten Chemokine der Vertebraten (DeVries et al. 2006). Dabei beträgt seine Sequenz-Homologie zwischen *Homo sapiens* und *Mus musculus* 99 %. Man unterscheidet heute sechs humane (α-ϕ) und drei murine (α-γ) Isoformen, die sich ausschließlich durch alternatives Spleißen im distalen C-Terminus unterscheiden (Yu et al. 2006). Je nach Isoform kodiert *Cxcl12*, das auf dem humanen Chromosom 10 und dem murinen Chromosom 6 liegt, für ein zwischen 89 (α) und 140 (δ) Aminosäuren langes Protein (inklusive eines 22 Aminosäure langen Signalpeptids; ncbi.nlm.nih.gov). In den hier vorgelegten Manuskripten wurde für *in situ* Hybridisierung eine *RNA*-Sonde verwendet, die sowohl CXCL12α, β also auch γ erkennt. *In vitro* Stimulationsexperimente wurden mit CXCL12α durchgeführt.

Während der C-Terminus zwischen den CXCL12-Isoformen variiert, ist der N-Terminus, über den die Bindung an die Rezeptoren CXCR4 und CXCR7 erfolgt, gleich. CXCR4 (auch LESTR, Fusin oder CD184 genannt) ist der einzig bekannte G-Protein-gekoppelte Rezeptor für CXCL12 und wurde als solcher 1996 identifiziert (Bachelerie et al. 2014, Bleul et al. 1996, Oberlin et al. 1996). Seine Struktur und Ligandenbindung wurden durch zahlreiche biochemische und biophysikalische Methoden detailliert charakterisiert (Bachelerie et al. 2014). Kristallstrukturanalysen von CXCR4 bestätigten seine heptahelikale Struktur (Abb.1) und zeigten einige Besonderheiten, wie beispielsweise die Orientierung der einzelnen Helices zueinander, das Fehlen der kurzen Helix VIII und der Palmitoylierungsstelle am C-Terminus (Bachelerie et al. 2014). Die CXCL12-Bindung an CXCR4 erfolgt in zwei Schritten (*„two-step" binding*, Crump et al. 1997). Hierfür befinden sich in den ersten 17 Aminosäuren des CXCL12 N-Terminus zwei essentielle Bindestellen: Stelle 1 am N-Terminus und eine weiter im Zentrum gelegene Stelle 2. Bei der CXCL12/CXCR4-Interaktion bindet die Stelle 2 des Chemokins an den N-Terminus von CXCR4 und die Stelle 1 in der Bindungstasche von CXCR4, was letztendlich zur Konformationsänderung und G-Protein-Aktivierung führt. Die nachgeschalteten Signalwege (Abb.1) teilt sich CXCR4 mit anderen typischen Chemokin-Rezeptoren. Der Rezeptor koppelt an ein Pertussis-Toxin-sensitives G_i-Protein (dies führt u. a. zur Adenylatzyklasen-Hemmung), stimuliert Phospholipase C (resultiert in einer Kalzium-Mobilisierung), aktiviert die MAP-Kinase (*mitogen-activated protein*)-Kaskade und den Phosphoinositid-3 Kinase (PI3K)-Signalweg (Busillo und Benovic 2007). Zusätzlich kommt es nach einer CXCL12-Stimulation zu Phosphorylierungen am CXCR4 C-Terminus, die von G-Protein-Rezeptor-Kinasen (GRK) und Protein-Kinase C (PKC) vermittelt werden (Busillo und Benovic 2007, Busillo et al. 2010, Mueller et al. 2013). Die Phosphorylierung wird von

β-Arrestin erkannt, wodurch es zu einer β-Arrestin-vermittelten Signaltransduktion und Internalisierung des CXCR4 kommt. Nach Internalisierung kann der Rezeptor abgebaut werden oder zurück zur Plasmamembran gelangen (Abb.1) (Busillo und Benovic 2007).

Manuskript II und andere Veröffentlichungen zeigen, dass CXCR4 unter langanhaltender Stimulation durch CXCL12 (homologe Aktivierung) verstärkt dem lysosomalen Abbau zugeführt und herunterreguliert wird (Sanchez-Alcaniz et al. 2011, Tarasova et al. 1998). Ein wichtiges Werkzeug, um Phosphorylierung und Regulation des CXCR4 untersuchen zu können, ist der Antikörper UMB-2 (Fischer et al. 2008). Das UMB-2-Epitop liegt im C-Terminus in den Aminosäureresten 343-352. Es wird von UMB-2 nur erkannt, wenn keine Phosphorylierung im Serin-Cluster 346-348 vorliegt. Da dieses Cluster sofort nach CXCL12-Stimulation phosphoryliert wird (Mueller et al. 2013), bindet UMB-2 nur an den inaktiven Rezeptor. In biochemischen und immunhistochemischen Analysen kann man so den Aktivierungszustand des Rezeptors nachvollziehen (Abb. 1, Manuskript I–III).

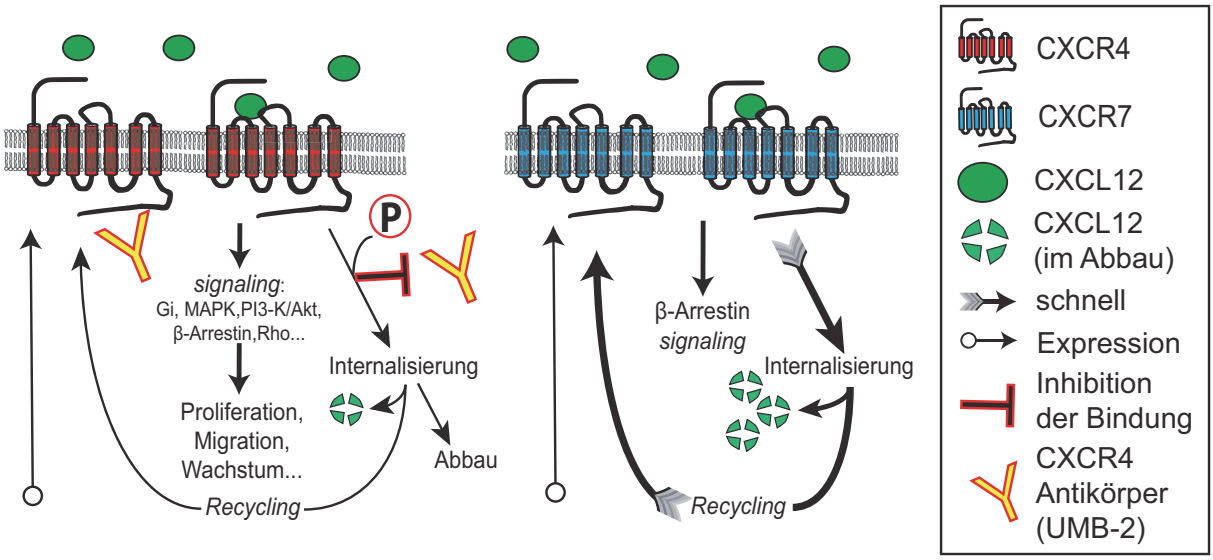

Abbildung 1 Schematische Darstellung des CXCL12/CXCR4/CXCR7-Systems. Links, CXCR4 (rot) wird exprimiert und zur Plasmamembran transloziert. In diesem inaktiven Zustand wird der Rezeptor vom UMB-2-Antikörper erkannt. Durch CXCL12-Bindung (grüner Punkt) wird CXCR4 aktiviert und führt über verschiedene Signalwege u. a. zu Proliferation, Wachstum und Migration von Zellen. Zusätzlich kommt es zur Phosphorylierung des CXCR4 am C-Terminus, wodurch die UMB-2-Bindung verhindert wird. Der Rezeptor wird internalisiert, anschließend abgebaut oder dem *Recycling* zugeführt. **Rechts**, CXCR7 (blau) zirkuliert liganden-unabhängig zwischen Plasmamembran und intrazellulären Kompartimenten. Dies kann durch CXCL12-Bindung beschleunigt ablaufen, wobei CXCL12 intrazellulär dem Abbau zugeführt wird (*scavenger*-Funktion). Außerdem ruft die CXCL12-Bindung an CXCR7 eine Aktivierung von β-Arrestin-vermittelten Signalwegen hervor.

CXCR7 wurde 2005 als zweiter CXCL12-Rezeptor entdeckt (Balabanian et al. 2005). Er ist unter den Chemokin-Rezeptoren zusammen mit CXCR4 evolutionär am stärksten konserviert (Bachelerie et al. 2014). Im Unterschied zu CXCR4, dessen einziger bekannter endogener Ligand CXCL12 ist, bindet CXCR7 zusätzlich das Chemokin CXCL11 (I-TAC). CXCR7 besitzt im Vergleich zu CXCR4 eine ca. 10-fach höhere Affinität zu CXCL12 (Balabanian et al. 2005, Burns et al. 2006). Allerdings führt die Ligandenbindung nicht zur Aktivierung von

G-Proteinen und zur Auslösung typischer Chemokin-Rezeptor-vermittelnder Signaltransduktionswege (Graham et al. 2012). Deshalb wird CXCR7 der Gruppe der atypischen Chemokin-Rezeptoren zugeordnet und nach aktueller Nomenklatur „atypischer Chemokin-Rezeptor 3“ (ACKR3) genannt. Um den Bezug zu den Manuskripten I–III zu wahren, wird in der vorliegenden Arbeit die Bezeichnung CXCR7 weiter verwendet.

CXCR7 zirkuliert kontinuierlich zwischen Plasmamembran und endosomalen Zellkompartimenten, wobei er seine Fracht dem lysosomalen Abbau zuführen kann. Dieses Zirkulieren läuft ligandenunabhängig ab, kann aber durch Liganden beschleunigt werden (Luker et al. 2010, Hoffmann et al. 2012, Naumann et al. 2010). Wenn CXCR7 sein Chemokin bindet, internalisiert, dem Abbau zuführt und selbst wieder an die Plasmamembran zurückkehrt, spricht man von *scavenging* (englisch für reinigen, wegfangen) (Abb.1). Durch diesen Prozess des *scavenging*/Wegfangens wird letztendlich die lokale Verfügbarkeit des Chemokins beeinflusst. So konnten die Manuskripte II, III und zahlreiche weitere Veröffentlichungen zeigen, dass das CXCL12-*scavenging* durch CXCR7 eine seiner wichtigsten biologischen Funktionen darstellt (Boldajipour et al. 2008, Burns et al. 2006, Luker et al. 2010, Sanchez-Alcaniz et al. 2011, Venkiteswaran et al. 2013). Ein wichtiges Molekül für die Vermittlung der CXCR7-*scavenger*-Funktion ist β-Arrestin. Es ist daran beteiligt, die intrazelluläre CXCR7-Sortierung zu steuern. Dabei unterstützt es nach Internalisierung des CXCL12/CXCR7-Komplexes das *Recycling* von CXCR7 vom späten Endosomen zurück zur Plasmamembran. Der internalisierte Ligand CXCL12 wird dagegen dem Abbau zugeführt (Mahabaleshwar et al. 2012). Des Weiteren führt die CXCL12/CXCR7-Interaktion zur β-Arrestin-vermittelten Aktivierung der MAP-Kinase-Kaskade (Abb.1) (Rajagopal et al. 2010, Wang et al. 2011).

2.2 Die Entwicklung neuronaler Strukturen unter dem Einfluss des Chemokins CXCL12

Das Chemokin CXCL12 und seine Rezeptoren CXCR4 und CXCR7 haben essentielle Aufgaben bei der Embryogenese. Dies drückt sich besonders durch den perinatalen Tod von Mäusen aus, bei denen eines der drei Gene entfernt (*ausgeknockt*) wurde (Nagasawa et al. 1998, Sierro et al. 2007, Zou et al. 1998). Die Letalität wird auf Entwicklungsfehler im blutbildenden und kardiovaskulären System zurückgeführt. Neben diesen Systemen trägt CXCL12 erheblich zur Entwicklung neuronaler Strukturen bei, wobei es kritische Aufgaben bei neuronaler Proliferation, Migration und Axon-Wachstum übernimmt (Deverman und Patterson 2009, Li und Ransohoff 2008, Stumm und Hollt 2007, Tiveron und Cremer 2008).

Die Rolle von CXCL12 bei der neuronalen Migration folgt dem Prinzip, dass CXCL12 als chemotaktischer Botenstoff auf neuronale Vorläuferzellen wirkt und dadurch deren Verteilung in einem Gewebe bzw. die Wanderung zu einer bestimmten Zielstruktur unterstützt. Dies trifft u. a. auf zerebrokortikale Interneurone (Manuskript I, II), Cajal-Retzius (CR)-Zellen (Abb. 2A), *Gonadotropin-Releasing*-Hormon-produzierende (GnRH)-Neurone (Manuskript III, Abb. 3), Körnerzell-Vorläufer in *Zerebellum* und *Gyrus dentatus*, Trigeminal- und Hinterwurzel-Ganglienzellen sowie olfaktorische Neurone zu (Deverman und Patterson 2009, Li und Ransohoff 2008, Stumm und Hollt 2007, Tiveron und Cremer 2008, Wang und Knaut 2014).

2.2.1 CXCL12 und die zerebrokortikale Entwicklung

2.2.1.1 Zusammensetzung des zerebralen Kortex

Der zerebrale Kortex ist an einer Vielzahl höherer kognitiver, emotionaler, sensorischer und motorischer Funktionen beteiligt. Er ist ein Bestandteil des Telenzephalons (Endhirns) und besteht bei Säugetieren aus sechs Schichten, die zytoarchitektonisch und funktionell unterscheidbar sind. Die kortikalen Neurone in diesen Schichten setzen sich aus ungefähr 80 % glutamatergen exzitatorischen Projektionsneuronen (Pyramidenzellen) und 20 % GABAergen inhibtorischen Interneuronen zusammen (Parnavelas 2000). Beide Klassen von Neuronen werden weiter in Untergruppen eingeteilt. Projektionsneurone werden dabei nach ihrer Position in den Schichten und ihrem Projektionsmuster unterschieden. So projizieren die meisten Schicht VI-Projektionsneurone zum Thalamus (kortikothalamische Verbindungen), wohingegen Projektionsneurone aus Schicht V in Verbindung mit Basalganglien, Mittel-, Hinterhirn und Rückenmark stehen (subzerebrale Verbindungen). Im Gegensatz dazu erhalten bedornte Sternzellen (*spiny stellate neurons*) der Schicht IV den überwiegenden Anteil ihrer Afferenzen vom Thalamus (thalamokortikale Axone [TCA]) und projizieren lokal in den zerebralen Kortex. Projektionsneurone der oberen Schichten II und III besitzen Verbindungen mit der kontra- und ipsilateralen Kortex-Hemisphäre (kortikokortikale Verbindungen) (Marin und Muller 2014). GABAerge Interneurone verschalten sich im Unterschied zu den Projektionsneuronen meist lokal. Man kann sie nach molekularen, morphologischen und physiologischen Kriterien in ca. 30 verschiedene Untergruppen aufteilen (DeFelipe et al. 2013). Auch hier gibt es bei einigen der Untergruppen die Tendenz, spezielle Schichten zu besiedeln. Martinotti-Zellen präferieren beispielsweise die Schichten II, III, V und VI,

während *double bouquet* Zellen eher in den Schichten II und III zu finden sind. Dahingegen sind Korbzellen gleichmäßig über die Schichten II–VI verteilt (Marin und Muller 2014).

2.2.1.2 Radiale und tangentiale Migration im sich entwickelnden Kortex

Die komplexe Zusammensetzung des zerebralen Kortex entsteht durch zahlreiche Prozesse, die intrinsische und extrinsische Netzwerke etablieren. Solche Prozesse umfassen Zellproliferation, Migration und Integration, was bezogen auf Neurone das Ausbilden von Verknüpfungen mit anderen Zellen über Dendriten und Axonen beinhaltet. Neurone entstehen in Proliferationszonen, die sich angrenzend zum Ventrikel befinden und als Ventrikulärzone (VZ) bezeichnet werden. Von dort aus verteilen sich die neugeborenen Neurone mittels radialer bzw. tangentialer Migration über das Nervensystem. Radiale Migration beschreibt die Zellwanderung senkrecht zur VZ und erfolgt meist entlang radialer Gliafasern. Tangentiale Migration verläuft parallel zur VZ und orthogonal zu den Gliafasern (Abb. 2A unten, blauer und grüner Pfeil) (Marin et al. 2010).

Bei der Entstehung des zerebralen Kortex sind diese beiden Migrationsarten essentiell. Im dorsalen Telenzephalon (*Pallium*) entstehen die Projektionsneurone aus asymmetrischer Zellteilung von radialen Gliazellen in der VZ oder durch symmetrische Zellteilung von basalen Vorläuferzellen (*intermediate progenitor cells*, IPC) in der Subventrikularzone (SVZ) (Marin und Muller 2014). Anschließend wandern sie radial entlang kortikaler Gliafasern, durch Schichten zuvor gebildeter Projektionsneurone nach außen, wodurch erst die Kortikalplatte (*cortical plate*, CP) und später die sechs Schichten des Kortex entstehen. Diese radiale Migration folgt also einem „*inside-out*“ Muster, bei dem „frühgeborene“ Neurone die unteren Schichten und „spätgeborene“ Neurone die oberen Schichten hervorbringen (Angevine und Sidman 1961, Rakic 1974). Während die Projektionsneurone lokal im sich entwickelnden Kortex entstehen, entstammen Interneurone dem ventralen Telenzephalon (*Subpallium*) und müssen erst eine lange Strecke tangential migrieren, bevor sie in ihre Zielregion im Kortex gelangen (Abb. 2A) (Marin et al. 2010). Das *Subpallium* besteht aus fünf proliferativen Zonen, den lateralen (LGE), medialen (MGE) und kaudalen (CGE) Ganglienhügeln (*ganglionic eminence*, GE) sowie der präoptischen Region (POA) und der *Septum*-Anlage. Kortikale Interneurone stammen je nach Subtyp aus MGE, CGE oder POA, wobei die drei Regionen im geschätzten Verhältnis von 6 : 3 : 1 zur gesamten kortikalen Interneuron-Population beitragen (Marin 2013).

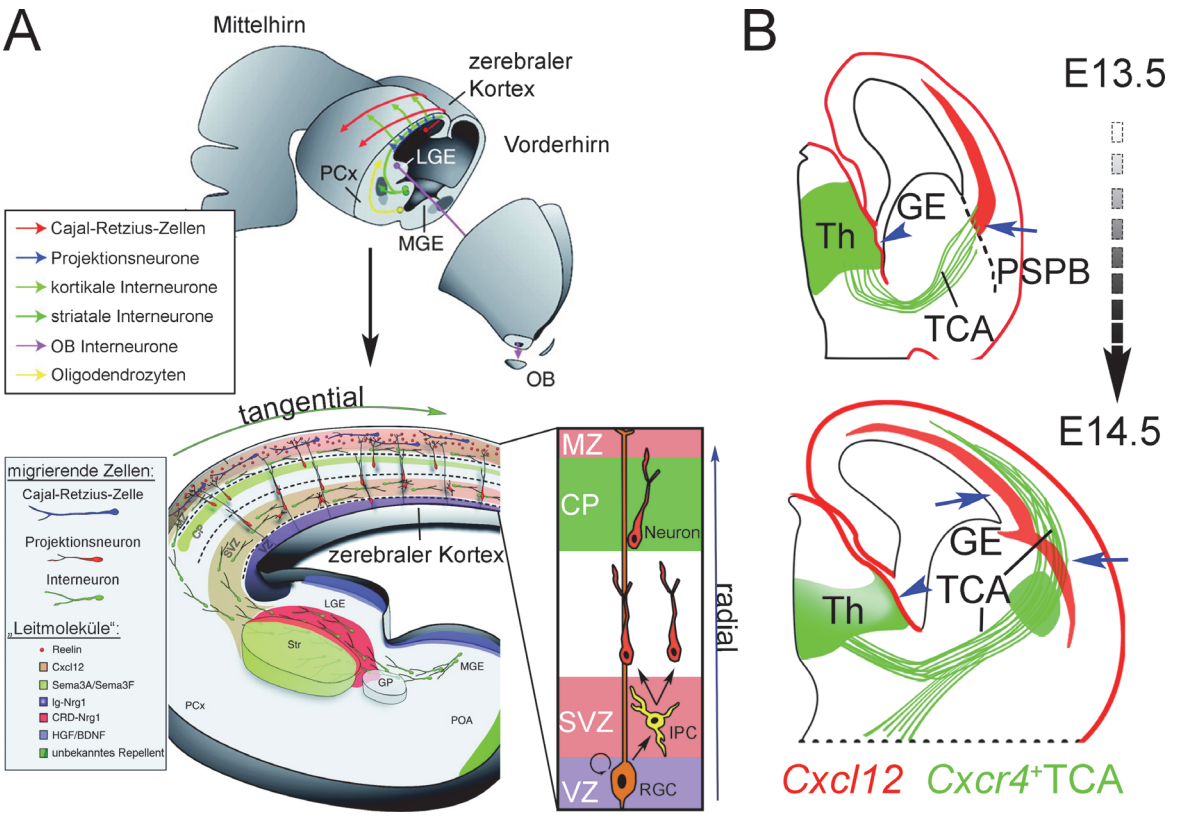

Abbildung 2 CXCL12 trägt zur zerebrokortikalen Entwicklung durch Koordination von Migration und Axon-Wachstum bei. A. Die Migrationsrouten unterschiedlicher Neurone werden von verschiedenen Molekülen beeinflusst (siehe Legende links). Kortikale Interneurone migrieren tangential (grüner Pfeil) über stereotype Routen in der MZ und der SVZ. Dabei werden sie von meningealem CXCL12 (rot) in der MZ und durch IPC-gebildetes CXCL12 (rot) in der SVZ gehalten. Kortikale Projektionsneurone entstehen durch Teilung radialer Gliazellen (RGC) und IPC und migrieren radial (blauer Pfeil) in Richtung CP. **B.** *Cxcr4*-positive thalamokortikale Axone (TCA, grün) wachsen ausgehend vom Thalamus (Th) ventral in die *Capsula interna* und erreichen die PSPB (gestrichelte Linie) zwischen E13 und E14. Die darauffolgende schnelle, latero-mediale Ausbreitung innerhalb des Kortex (gestrichelter Pfeil) wird durch die wachstumsfördernden Eigenschaften von CXCL12 (rot) unterstützt. Die blauen Pfeile symbolisieren neuronales CXCL12 und die Pfeilspitzen nicht-neuronales (meningeales) CXCL12. Zusätzliche Abkürzungen: IPC, *intermediate progenitor cell*; GP, *Globus pallidus*; OB, *Bulbus olfactorius*; PCx, piriformer Kortex; Str, Striatum. *A*, erweiterte Illustration aus Marin et al. 2010.

2.2.1.3 CXCL12 steuert die tangentiale Migration kortikaler Interneurone

Der Weg der zerebrokortikalen Interneurone kann in drei Etappen gegliedert werden: Erstens den Weg vom *Subpallium* zum *Pallium*, zweitens die Verteilung im Kortex über stereotype Routen und drittens die Integration in eine spezifische kortikale Schicht. Während die initiale Migration vom *Subpallium* zum *Pallium* ohne CXCL12-Beteiligung abläuft, ist dessen Beitrag zur intrakortikalen Verteilung von entscheidender Bedeutung (Marin 2013). Zur Zeit ist CXCL12 das einzig bekannte Molekül, welches die Interneuron-Migration auf ihren Routen in der Marginalzone (MZ) und SVZ vermittelt (Marin 2013). Die Routen werden dabei durch seine Expression, geringe Diffusion *in vivo* (aufgrund hoher Affinität zu Heparansulfaten) und letztendlich seiner chemoattraktiven Wirkung definiert (Marin 2013). Zerebrokortikales CXCL12 wird von nicht-neuronalen meningealen Zellen und neuronalen Zellen, die sich in SVZ und SP befinden, exprimiert (Manuskript II, Stumm et al. 2007, Stumm et al. 2003, Tham et al. 2001, Tiveron et al. 2006). Interessanterweise kommt es durch

eine fehlerhafte CXCL12-Expression meist zu einer abnormalen Interneuron-Migration. Demnach folgt einer meningealen Reduktion von *Cxcl12* in einem hypomorphen *Foxc1*-Mausmodell eine Abnahme von migrierenden Interneuronen in der MZ (Zarbalis et al. 2012). Dabei scheint der Transkriptionsfaktor FOXC1 durch Bindung eines regulatorischen Elements im *Cxcl12*-Intron3 für eine hohe CXCL12-Expression in den Vorderhirn-Meningen notwendig zu sein. Auch in der SVZ kommt es durch Transkriptionsfaktoren, die spezifisch in neuronalen Projektionsneuron-Vorläufern exprimiert werden, zu einer Modulation der CXCL12-Expression. So zeigen *Pax6*- und *Tbr2-knockout*-Mäuse neben einer fehlerhaften Kortexentwicklung auch den Verlust von *Cxcl12* in der SVZ. Zusätzlich wurde eine intrakortikale Verschiebung der Interneurone von SVZ in höhere Schichten (CP und MZ) beobachtet. Die Autoren erklären diese Ablenkung der migrierenden Interneurone durch den Verlust von CXCL12 in der SVZ und bestehender CXCL12-Expression in den Meningen (Sessa et al. 2010, Tiveron et al. 2006). Daraus ergab sich die Hypothese, dass CXCL12 aus den Vorläufern kortikaler Projektionsneurone GABAerge Interneurone rekrutiert. Diese Hypothese wurde im Manuskript I erstmals kausal belegt und damit der Einfluss sekundärer Effekte (z. B. fehlerhafte Kortexentwicklung und Reduktion anderer migrationsrelevanter Moleküle) (Elsen et al. 2013), die durch den Verlust dieser Transkriptionsfaktoren entstehen, ausgeschlossen: Nach *Tbr2-Cre*-vermitteltem (konditionalem) Ausschalten von *Cxcl12* in basalen Vorläuferzellen (IPC) kommt es zu einer Interneuron-Migrationsverschiebung von unteren zu oberen Kortexschichten und intrakortikalen Verteilungsdefiziten entlang der tangentialen latero-medialen Achse. Folglich scheint IPC-produziertes CXCL12 für die effiziente Interneuron-Verteilung entlang der SVZ essentiell zu sein.

2.2.1.4 CXCR4 und CXCR7 sind für die Interneuron-Migration essentiell

Die chemoattraktive Wirkung von CXCL12 ist während der Interneuron-Migration von seinen Rezeptoren CXCR4 und CXCR7 abhängig. Dabei werden beide Rezeptoren von Interneuronen exprimiert und sind für die intrakortikale Migration essentiell (Manuskript II, Sanchez-Alcaniz et al. 2011, Stumm et al. 2003, Wang et al. 2011). In CXCR4-defizienten Embryonen verlassen kortikale Interneurone ihre Hauptrouten in der MZ und SVZ und akkumulieren verfrüht in der CP (Manuskript II, Lopez-Bendito et al. 2008, Stumm et al. 2003). Postnatale Analysen von interneuron-selektiven CXCR4-Nullmutanten verweisen auf einen entscheidenden Beitrag von CXCR4 und CXCL12 bei der pan-kortikalen und schichtspezifischen Verteilung der Interneurone (Li et al. 2008, Tanaka et al. 2010). 2011 zeigten zwei Publikationen, dass auch der Verlust von CXCR7 zu einer ähnlichen

Fehlverteilung der Interneurone innerhalb des Kortex führt (Sanchez-Alcaniz et al. 2011, Wang et al. 2011). Dies wirft die Frage auf, wie diese Ähnlichkeit im Phänotyp trotz unterschiedlicher Rezeptoreigenschaften von CXCR4 und CXCR7 zustande kommt.

Sowohl in CXCR4- als auch in CXCR7-Mutanten migrieren Lhx6-positive Interneurone weniger tangential und häufiger radial. Dies führt zu einer Anhäufung der Interneurone in der CP und zur Abnahme in der MZ und SVZ. Allerdings wurden auch Unterschiede im Migrationsverhalten in den beiden Mutanten beobachtet: CXCR4-defiziente Zellen waren beweglicher, wohingegen CXCR7-defiziente Zellen weniger beweglich bezogen auf die Wildtyp-Kontrolle waren (Wang et al. 2011). Dies lässt vermuten, dass die CXCL12-Bindung an CXCR4 oder CXCR7 unterschiedliche Prozesse und Signalwege auslöst. Die CXCL12/CXCR4-Interaktion aktiviert den G-Protein-Signalweg und lenkt die Interneurone in Richtung der CXCL12-Quelle (Lysko et al. 2011). CXCL12-vermittelte Aktivierung von CXCR7 moduliert hingegen den MAP-Kinase-Signalweg über β-Arrestin (Wang et al. 2011). Nach diesem Konzept regulieren CXCR4 und CXCR7 die Interneuron-Wanderung über verschiedene Signalkaskaden.

Ein weiteres Konzept kommt von der Arbeitsgruppe um Prof. Ralf Stumm. Dabei zeigt die Publikation Sánchez-Alcañiz et al. (2011), dass es bei migrierenden CXCR7-defizienten Interneuronen zum Verlust des CXCR4-Proteins kommt (trotz normaler *Cxcr4*-mRNA-Expression). Warum sollte CXCR4 unter CXCR7-Abwesenheit verschwinden? In migrierenden Interneuronen liegt CXCR7 meist intrazellulär vor. Er zirkuliert dabei zwischen dem Zellinneren und der Plasmamembran und ist ständig an der Bindung und Internalisierung von CXCL12 beteiligt (Sanchez-Alcaniz et al. 2011, Luker et al. 2010, Hoffmann et al. 2012, Naumann et al. 2010). Sánchez-Alcañiz et al. (2011) konnten zeigen, dass sich CXCL12 ohne CXCR7 im Kortex anreichert. Normalerweise kommt es durch CXCL12-Bindung an CXCR4 neben der G-Protein-Aktivierung zu einer Rezeptorinternalisierung und zum Rezeptorabbau (Kolodziej et al. 2008, Tarasova et al. 1998, Marchese und Benovic 2001). Da zu hohe CXCL12-Spiegel in der CXCR7-Mutante vorliegen, sollte dies gesteigerte CXCR4-Endozytose und –Degradation auslösen. Daraus lässt sich ableiten, dass CXCR7 die CXCL12-Konzentration feintitriert und dadurch die angemessene CXCL12-Menge für die CXCR4-Aktivierung reguliert. Demzufolge stellte sich Manuskript II die Frage, ob CXCR7 über seine CXCL12-*scavenger*-Aktivität CXCR4 gegen eine CXCL12-vermittelte Hyperaktivierung und Herunterregulation schützt. Es wurde gezeigt, dass bereits die Einzel-Nullmutanten von CXCR4 und CXCR7 ausreichend sind, um den Interneuron-Migrationsdefekt der vollen signaldefizienten Mutante (*Cxcl12*$^{-/-}$) hervorzubringen.

Weiterführend wurden Hinweise für die CXCL12-*scavenger*-Aktivität von CXCR7 in migrierenden Interneuronen gefunden. Lebendzellbeobachtung mit doppeltransgenen *Cxcr7*-GFP-Reporter- und CXCL12-RFP-Fusionsprotein-Embryonen zeigten erstmals, dass CXCL12-RFP von CXCR7-exprimierenden Interneuronen aufgenommen wird. Ohne CXCR7 kam es zur starken Abnahme von aufgenommenem und akkumuliertem CXCL12-RFP. Die verringerte CXCL12-Aufnahme führt zur kortikalen Anreicherung des Chemokins (Sanchez-Alcaniz et al. 2011), aber resultiert hieraus auch der Verlust des CXCR4-Proteins in CXCR7-defizienten Embryonen? Durch *in vitro* und *in vivo* Experimente wurde im Manuskript II belegt, dass die starke Abnahme des CXCR4-Proteins in CXCR7-Mutanten durch CXCL12-vermittelte Überaktivierung von CXCR4 herbeigeführt wird. Damit ergibt sich das folgende Modell für die Migration kortikaler Interneurone: Während die CXCL12/CXCR4-Interaktion hauptsächlich die Chemotaxis vermittelt, schützt CXCR7 über seine CXCL12-*scavenger*-Aktivität CXCR4 vor einer CXCL12-vermittelten Hyperaktivierung und Herunterregulation.

2.2.1.5 Der Wechsel von tangentialer zu radialer Migration

Interneurone erfassen ihre molekulare Umwelt über ihren nach vorne gerichteten Leitfortsatz (englisch *leading process*). Dabei kommt es zu verzweigenden Bewegungen am Leitfortsatz, die man als *branching* bezeichnet. Hierüber wird die Orientierung des Neurons als Antwort auf Leitstoffe gesteuert (Marin et al. 2010). CXCL12 ist solch ein Leitstoff, da beispielsweise das *branching* und die Geschwindigkeit der Interneurone von der CXCL12/CXCR4-Signaltransduktion abhängen. So kommt es durch Aktivierung von $G_{\alpha i}$ zur Absenkung des cAMP-Spiegels, was – vermittelt über die spezifische Spaltung von Aktin-Verzweigungen und Mikrotubuli-Stabilisierung – zu erhöhter Wanderungsgeschwindigkeit und verringertem *branching* führt (Lysko et al. 2011, Lysko et al. 2014). Diese Prozesse finden vor allem entlang tangentialer Migrationsrouten mit hohen CXCL12-Konzentrationen statt und führen dadurch zur Interneuron-Verteilung über den zerebralen Kortex. Nach einer bestimmten Reifungs- und Migrationszeit wechseln die Interneurone von tangentialer zu radialer Migration und besiedeln so die kortikalen Schichten. Welcher Mechanismus hinter diesem Wechsel steht, ist noch nicht bekannt (Marin 2013). Da der Verlust des Ansprechens auf CXCL12 mit dem Wechsel von tangentialer zu radialer Migration korreliert, ist dies derzeit die attraktivste Hypothese für diesen Prozess (Marin 2013, Li et al. 2008). Hierfür spricht auch, dass CXCL12-, CXCR4- und CXCR7-Nullmutanten eine verfrühte radiale Migration und Akkumulation der Interneurone in der CP zeigen (Manuskript II). Zusätzlich scheinen die Interneurone einer hierarchischen Abfolge von Leitmolekülen zu folgen. Unter dem Einfluss

von CXCL12 meiden Interneurone die CP, wohingegen der Verlust der CXCL12-Antwort, vermittelt über ein unbekanntes Leitmolekül, zur CP-Invasion führt (Marin 2013).

2.2.1.6 CXCL12 und die Entwicklung thalamokortikaler Axone

Die neuronale Verbindung zwischen Thalamus und Kortex nennt man thalamokortikale Axone (TCA). Diese Verknüpfung ist für die Wahrnehmung der Umwelt essentiell, da fast alle Sinnesorgane (Ausnahme ist der Geruchssinn) ihre Information zuerst an den Thalamus senden, worauf sie dort prozessiert und dann über thalamokortikale Axone an den Kortex weitergeleitet werden. Bei ihrer Entwicklung müssen die thalamokortikalen Axone einen langen Weg mit intermediären Zielen zurücklegen. Ähnlich dem Leitfortsatz der Interneurone haben diese Axone einen Wachstumskegel, der sich anhand chemoattraktiver bzw. –repulsiver Moleküle entlang definierter Routen orientiert (Molnar et al. 2012). Dabei wachsen thalamische Axone ventral aus dem Thalamus, meiden den Hypothalamus, wachsen geleitet durch Korridorzellen in die *Capsula interna* und erreichen in der Maus die *Pallium-Subpallium*-Grenze (PSPB) zwischen den Embryonaltagen (E) 13 und 14 (Abb. 2B) (Manuskript I, Molnar et al. 2012).

Es gibt zahlreiche Moleküle, welche die Axone vom Thalamus zum *Subpallium* leiten und dabei bereits eine topographische Organisation von bestimmten Thalamuskernen mit definierten Kortexgebieten vorselektieren (Lopez-Bendito und Molnar 2003, Molnar et al. 2012). Weniger ist über die Koordination des Wachstums der thalamischen Axone nach Überquerung der PSBP und innerhalb des zerebralen Kortex bekannt. Nach Eintritt in den Kortex zwischen E13 und E14 breiten sich die thalamischen Axone in der kortikalen Intermediärzone (IZ) über den Kortex aus (Abb. 2B). Dabei erreichen die Axone ihre kortikale Region, bevor ihre eigentlichen kortikalen Zielneurone angelegt sind, was sie zu einer Warteperiode in der SP veranlasst (in Nagern E16-E19) (Lopez-Bendito und Molnar 2003). Demnach ist es wahrscheinlich, dass es im sich entwickelnden Kortex Moleküle gibt, die das Einwachsen thalamischer Axone und die Entstehung der kortikalen Zielneurone koordinieren.

Ein erster Hinweis auf solch eine molekulare Interaktion brachte die Untersuchung des Slit/Robo-Systems (Mire et al. 2012). So konnte gezeigt werden, dass die intrakortikale Wachstumsdynamik vermittelt über das Robo/Slit-System von thalamischer Spontanaktivität abhängt. Dabei korreliert schnelles Axon-Wachstum in einer frühen Periode (E12-E14) mit hoher, spontaner Kalziumfrequenz und langsames axonales Wachstum in einer späten Phase (E16-E17) mit niedriger Kalzium-Spontanaktivität. Die Übersetzung von Spontanaktivität auf

Axon-Wachstum wird auf die Expression des Slit1 Rezeptors Robo1, der als intrinsische „Bremse“ auf die thalamischen Axone wirkt, zurückgeführt (Mire et al. 2012). Die hohe spontane Kalziumfrequenz in der frühen Periode hält die Robo1-Spiegel im Thalamus gering, während in der späten Phase die Kalziumfrequenz sinkt und die Robo1-Expression steigt. Diese erhöhte Robo1-Expression ermöglicht eine gesteigerte Interaktion mit seinem Liganden Slit1, welcher von reifenden kortikalen Projektionsneuronen exprimiert wird. Die Slit1/Robo1-Interaktion führt zur Verlangsamung der thalamokortikalen Axone (Mire et al. 2012).

Das Manuskript I beschäftigt sich mit der Frage, ob CXCL12 ein weiteres Molekül ist, welches die Dynamik der thalamokortikalen Axone beeinflusst. Außerhalb des Gehirns wurde der Einfluss von CXCL12 auf axonales Wachstum bereits beschrieben (Deverman und Patterson 2009, Li und Ransohoff 2008, Stumm und Hollt 2007). CXCL12 senkt beispielsweise die repulsive Aktivität von Semaphorin3A (Sema3A), Sema3C und Slit2 und moduliert dadurch das Axon-Wachstum von retinalen Ganglienzellen, sensorischen und sympathischen Hinterwurzelganglienzellen (Chalasani et al. 2003, Chalasani et al. 2007). Der Einfluss von CXCL12 auf die Ausbildung der thalamokortikalen Verknüpfung ist unbekannt. Das Manuskript I zeigt, dass thalamische Neurone und thalamokortikale Axone den CXCL12-Rezeptor CXCR4 tragen. CXCL12 wird von Meningen, die dem Thalamus und Kortex angrenzen, und von kortikalen Projektionsneuron-Vorläufern (IPC) exprimiert. Da thalamokortikale Axone CXCR4 besitzen und CXCL12 in ihrer Zielregion – dem Kortex – exprimiert wird, stellte sich die Frage, ob CXCL12 die Entwicklung der thalamokortikalen Fasern beeinflusst. Mittels genetischer Mausmodelle zeigt Manuskript I, dass thalamokortikale Axone in Abwesenheit von CXCL12 langsamer in den Kortex einwachsen. Im Gegensatz hierzu führt CXCL12-Überexpression zu beschleunigtem Axon-Wachstum bis hin zu verfrühter CP-Invasion. In Analogie zu der Migration kortikaler Interneurone (siehe oben) hängt auch das Wachstum der thalamokortikalen Axone von CXCL12, welches von kortikalen Projektionsneuron-Vorläufern exprimiert wird, ab.

Zusammenfassend zeigt dies die entscheidende Rolle von nicht-neuronalem (meningealem) und neuronalem CXCL12 bei der Entwicklung des zerebralen Kortex. Dabei unterstützt CXCL12 die frühe Entwicklung kortikaler Netzwerke durch Koordination von Bestandteilen, die extrakortikalen Ursprungs sind.

2.2.2 CXCL12 beeinflusst die lange Wanderung von GnRH-Neuronen

GnRH-Neurone entstehen außerhalb des zentralen Nervensystems (ZNS) im Vomeronasalorgan (VNO) und legen eine lange Wanderung bis zu ihrem finalen Ziel im Hypothalamus zurück. Während dieser Migration navigieren sie entlang VNO- bzw. olfaktorischer Axone und orientieren sich an einer Vielzahl von Faktoren (Schwarting et al. 2007). Bei der initialen Wanderung vom VNO zur *Lamina cribrosa* ist CXCL12 essentiell. Es wird im nasalen Mesenchym von nicht-neuronalen Zellen in einem rostro-kaudalen Gradienten exprimiert und leitet dadurch CXCR4-positive GnRH-Neurone in Richtung basales Vorderhirn (Abb.3) (Manuskript III, Schwarting et al. 2006). CXCR4- und CXCL12-defiziente Mäuse haben, neben einer reduzierten Gesamtzahl, eine deutlich verringerte Anzahl von GnRH-Neuronen im Hypothalamus (Manuskript III,(Schwarting et al. 2006). Dies lässt auf eine Rolle von CXCL12/CXCR4 bei dem Überleben und der Migration von GnRH-Neuronen schließen. Daran anknüpfend zeigt Manuskript III, dass der zweite CXCL12-Rezeptor CXCR7 modulierend auf diese Prozesse Einfluss nimmt. Im Unterschied zu kortikalen Interneuronen, die CXCR4 und CXCR7 besitzen, exprimieren GnRH-Neurone nur CXCR4. CXCR7 wird hingegen von Zellen (nicht Axonen) entlang oder nahe der GnRH-Neuron-Migrationsroute exprimiert. Da *Cxcr7*-k*nockout*-Mäuse ebenfalls einen GnRH-Neuron-Migrationsdefekt zeigen, scheint dies ein nicht-zellautonomer Mechanismus zu sein. Nachfolgende Experimente zeigten, dass CXCR7 als CXCL12-*scavenger* fungiert und dadurch die lokale Verfügbarkeit von CXCL12 im nasalen Kompartiment beeinflusst. Ohne CXCR7 kommt es zur erhöhten CXCL12/CXCR4-Interaktion, was wiederum zur Internalisierung und Herunterregulation von CXCR4 führt. Durch die CXCR4-Reduktion und die Umverteilung von CXCL12 können die GnRH-Neurone nicht mehr effizient von rostral nach kaudal migrieren und verbleiben im nasalen Kompartiment.

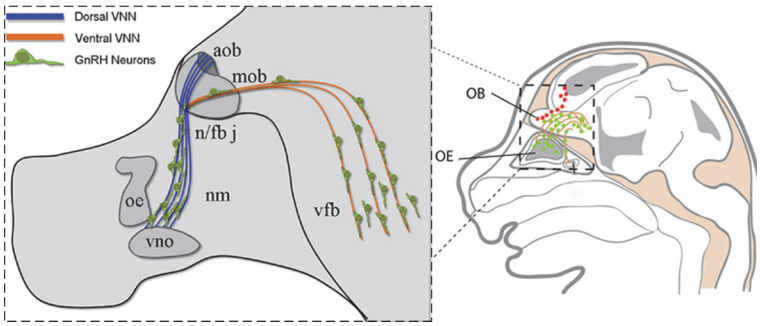

Abbildung 3 Schematische Übersicht über die Migration von GnRH-Neuronen. Sagittale Sicht auf den Kopf eines Mausembryos (links: simplifizierte Vergrößerung des vorderen Kopfes). GnRH-Neurone (grün) entstehen im VNO, migrieren entlang axonaler Fasern (blau und orange) durch das nasale Mesenchym (NM), durchqueren die *Lamina cribrosa* (n/fb J) um sich anschließend im ventralen Vorderhirn (vfb, Hypothalamus) zu integrieren. aob und mob, akzessorischer und „Haupt"- *Bulbus olfactorius*, OB, *Bulbus olfactorius*; OE, olfaktorisches Epithel; VNN, VNO-Nerv. (Zusammengesetzte Abbildung nach: Messina und Giacobini 2013, Suarez et al. 2012)

3 Ziele der Arbeit

Hintergrund:
Das Chemokin CXCL12 steuert unter anderem Proliferation, Migration, Wachstum und Differenzierung von Nervenzellen. Die vorliegende kumulative Arbeit untersucht, wie die Rezeptoren CXCR4 und CXCR7 diese CXCL12-Funktionen vermitteln. Die Manuskripte I und II betrachten dazu die Entwicklung des zerebralen Kortex und wie das Zusammenspiel von CXCL12, CXCR4 und CXCR7 die Migration GABAerger Interneurone und das Wachstum thalamokortikaler Axone beeinflusst. Manuskript III zeigt, dass CXCR7 die Migration von GnRH-Neuronen durch Steuerung der lokalen CXCL12-Verfügbarkeit moduliert.

Ziele von Manuskript I: Die thalamische Expression von CXCL12, CXCR4 und CXCR7 sollte erstmals mittels *in situ* Hybridisierung (ISH) und Immunohistochemie (IHC) dargestellt werden. Hiernach galt es die Aktivierung von CXCR4 durch CXCL12 in thalamischen Neuronen zu überprüfen und zu untersuchen, ob CXCL12 die Entwicklung des Thalamus beeinflusst. Des Weiteren sollte die Rolle von nicht-neuronalem (meningealem) und neuronalem CXCL12 bei der Steuerung des thalamokortikalen Axon-Wachstums und der Interneuron-Migration an konditionalen *Cxcl12-knockout-Mäusen* untersucht werden.

Ziele von Manuskript II: Im Vorfeld der Arbeit wurde berichtet, dass die Interneuron-Migration von CXCL12 und von den beiden CXCL12-Rezeptoren CXCR4 und CXCR7 gesteuert wird (Sanchez-Alcaniz et al. 2011, Stumm et al. 2003, Wang et al. 2011). Dabei vermittelt die CXCL12/CXCR4-Signaltransduktion hauptsächlich die Chemotaxis der Interneurone entlang der Hauptmigrationsrouten in der MZ und SVZ. Für CXCR7 wurde vermutet, dass er diesen Prozess moduliert, indem er die lokale CXCL12-Verfügbarkeit kontrolliert (*scavenger*-Funktion des CXCR7). Ferner wurde vorgeschlagen, dass CXCR7 über β-Arrestin die MAP-Kinase aktiviert und dadurch als signalgebender Rezeptor in die Interneuron-Migration eingreift. Im Manuskript II sollte erstmals ein direkter Vergleich der Interneuron-Migrationsphänotypen in *Cxcl12*$^{-/-}$, *Cxcr4*$^{-/-}$ und *Cxcr7*$^{-/-}$ Embryonen hergestellt werden. Die CXCR7-*scavenger*-Funktion sollte mittels *ex vivo* Lebendzellbeobachtung und Immunhistochemie überprüft werden. Dabei sollten CXCL12-RFP-transgene Mäuse zur Visualisierung der CXCR7-vermittelten CXCL12-Aufnahme in Interneurone genutzt werden.

Außerdem sollte überprüft werden, ob der Verlust des CXCR4-Proteins im Gehirn von CXCR7-defizienten Mausembryonen durch CXCL12 bewirkt wird.

Ziele von Manuskript III: Das Expressionsmuster von CXCR7 sollte erstmals in der Nasenregion embryonaler Mäuse charakterisiert werden. Auch hier sollten CXCL12-RFP-transgene Mäuse eingesetzt werden, um die Interaktion von CXCL12 und seinen Rezeptoren im Gewebeverband darzustellen. Durch Untersuchung von *Cxcr7*$^{-/-}$ Embryonen sollte geklärt werden, ob das Fehlen des CXCR7-Rezeptors zum Verlust des CXCR4-Proteins in GnRH-Neuronen führt und ob dies von einem Migrationsdefekt der Zellen begleitet wird.

Zusammenfassend sollte in Manuskript I erstmals untersucht werden, ob CXCL12 an der Embryonalentwicklung des Thalamus beteiligt ist. Die Manuskripte II und III konzentrieren sich darauf, die atypische Chemokin-Rezeptor-Funktion des CXCR7 (ACKR3) im Prozess der neuronalen Migration herauszuarbeiten.

4 Manuskripte

4.1 Manuskript I – Intermediate Progenitors Facilitate Intracortical Progression of Thalamocortical Afferents and Interneurons through CXCL12 Chemokine Signaling

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Ralf Stumm und Philipp Abe haben das Projekt entwickelt. Philipp Abe hat die Experimente durchgeführt, die Daten ausgewertet und das Manuskript zusammen mit Ralf Stumm entworfen. Philipp Abe und Zoltán Molnár haben die DiI *tracing* Studie vollzogen. Ralf Stumm war in die Datenanalyse involviert und hat das finale Manuskript geschrieben. Sebastian J. Arnold, Yi-Shiuan Tzeng und Dar-Ming Lai haben die Mäuse zu Verfügung gestellt (*Tbr2*$^{Cre/+}$, *Cxcl12*-flox) und unterstützend am Manuskript gearbeitet.

Intermediate Progenitors Facilitate Intracortical Progression of Thalamocortical Afferents and Interneurons through CXCL12 Chemokine Signaling

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RUNNING TITLE: Cortical basal progenitors signal via CXCL12

ABSTRACT

Glutamatergic principal neurons, GABAergic interneurons and thalamocortical afferents (TCAs) are essential elements of the cerebrocortical network. Principal neurons originate locally from radial glia and intermediate progenitors (IPCs) whereas interneurons and TCAs are of extrinsic origin. Little is known how the assembly of these elements is coordinated. C-X-C motif chemokine 12 (CXCL12), which is known to guide axons outside the neural tube and interneurons in the cortex, is expressed in the meninges and IPCs. We dissected the influence of IPC-derived CXCL12 on TCAs and interneurons by showing that *Cxcl12* ablation in IPCs, leaving meningeal *Cxcl12* intact, attenuates intracortical TCA growth and disrupts tangential interneuron migration in the subventricular zone. Mechanistically, our data suggest that intracortical TCA growth is regulated by crosstalk between growth-promoting CXCL12 and repellent cortical plate-derived Slit1. Thus, a CXCL12 signal from IPCs links cortical neurogenesis to the progression of TCAs and interneurons spatially and temporally.

INTRODUCTION

The cerebral cortex exerts perceptual, motor and cognitive functions including emotional processing. Cortical neurons roughly consist of 80% glutamatergic principal neurons (PNs) and 20% GABAergic interneurons (Parnavelas, 2000). Thalamocortical afferents (TCAs) terminating on layer IV PNs convey sensory information to the cortex and represent its major excitatory input. Neurodevelopmental defects that disturb the balance of these excitatory and inhibitory systems are thought to underlie epilepsy and mental disorders including schizophrenia, personality disorders and autism spectrum disorders. Thus, elucidating the mechanisms that govern the assembly of the cortical network is central to understanding causes and pathogenesis of mental illnesses (Rubenstein, 2011).

Cortical PNs are generated locally while cortical interneurons are of subpallial origin. In mice, most interneurons enter the cortex after embryonic day 12 (E12). They migrate preferentially on characteristic routes including the cortical subventricular zone (SVZ) and marginal zone (MZ) (Wonders and Anderson, 2006). Individual interneurons spend days migrating in the cortex and perform random movements. This unusual behavior is thought to disperse the cells before they integrate into the network (Lopez-Bendito et al., 2008; Tanaka et al., 2009). Like interneurons, TCAs travel long distances before reaching their destination. Murine TCAs navigate through the ventral telencephalon for two days and reach the cortex around E14. Until E15.5, they traverse the cortex in the SVZ and intermediate zone (IZ) to form contacts with the subplate, where they pause for several days before growing into the cortical plate (Lopez-Bendito and Molnar, 2003; Mire et al., 2012). Thus, the peak of cortical neurogenesis, taking place from E12 to E16 in mice (Hevner et al., 2004), coincides with intracortical TCA growth and interneuron dispersion.

Cortical basal/intermediate progenitor cells (IPCs) generate a significant proportion of PN (Kowalczyk et al., 2009; Vasistha et al., 2014) and enable evolutionary expansion of the cortex by amplification of cell numbers (Martinez-Cerdeno et al., 2006). Sufficient interneurons and TCAs have to be integrated into the fast growing cortex, demanding signal mechanisms that coordinate PN production with progression of invading elements. Several findings suggest that the chemokine CXCL12 (stromal cell-derived factor-1) via its receptors CXCR4 and CXCR7 acts as such a coordinating signal. First, in addition to the meninges, CXCL12 is highly expressed in IPCs and guides migrating interneurons in the cortex (Stumm et al., 2003; Tiveron et al., 2006; Stumm and Hollt, 2007; Lysko et al., 2011; Sanchez-Alcaniz et al., 2011; Wang et al., 2011; Abe et al., 2014). Second, we observed that CXCR4 is highly

expressed in the forming thalamus and TCAs. In analogy to the CXCL12/CXCR4 axis defining the initial trajectories of motor axons growing out of the neural tube (Lieberam et al., 2005), CXCL12 might similarly by involved in the formation of the thalamocortical projection.

Despite the well-documented role of CXCL12 in cortical development, the specific contribution of IPC-derived CXCL12 has not been dissected. In this report, we employed explant experiments and a wealth of histochemical and fluorescent dye labeling technology in a series of mouse genetic models including conditional *Cxcl12* ablation in IPCs. We thus provide conclusive evidence that CXCL12, emanating from IPCs, promotes intracortical TCA growth and interneuron migration in the SVZ.

MATERIALS & METHODS

Animals

Animal procedures were in accordance with German and EU guidelines. *Cxcr4*-GFP and CXCL12-RFP mice (Bhattacharyya et al., 2008) were on CD1 background; $Cxcl12^{+/-}$ (Nagasawa et al., 1996), $Cxcl12^{LoxP}$ (Tzeng et al., 2011) and $Tbr2^{+/Cre}$ mice (Costello et al., 2011) were on C57BL/6J background.

Histology and DiI tracing

Established *in situ* hybridization and immunohistochemical procedures with previously characterized probes and antibodies were used (Stumm et al., 2002; Cho et al., 2007; Sanchez-Alcaniz et al., 2011; Suzuki-Hirano et al., 2011). A *Sox2* probe (GI:127140985, nucleotides 418 – 1681) was generated by RT-PCR cloning. Primary antibodies are listed in the table 1. Axon tracing was performed as described (Molnar et al., 1998).

Thalamic explant cultures

Thalami were prepared from E13.5 *Cxcr4*-GFP^{+} brains and cut into 300 x 300 µm pieces using a McIlwain tissue copper. Explants were placed onto collagen-coated coverslips, embedded in collagen (3 mg/ml, BD Biosciences) and cultured in Neurobasal Medium (Thermo Fisher Scientific) supplemented with 2 mM Glutamine (PAA) and 100 units/ml Pen-Strep (PAA). CXCL12 (Peprotech) and recombinant mouse Slit1 (R&D Systems) were added immediately after plating. Native GFP was imaged after 48 h in fixed explants.

Image acquisition and analysis

Confocal images were captured with an LSM510 Meta; other micrographs were taken using an Axio Imager A1 (Zeiss). Throughout the study, *Cxcl12*$^{-/-}$ mice were compared to control littermates (*Cxcl12*$^{+/+}$ or *Cxcl12*$^{+/-}$). All measurements (thalamus area, intracortical TCA length, expression levels, cell counts) were normalized to mean values of litter controls. The area of the developing thalamus was determined based on *Gbx2*, *Sox2* and *Slc17a6* patterns using ImageJ. Intracortical TCA length was determined using ImageJ as illustrated in *Results* (Figure 5W). Quantification of interneuron distribution in the cortex and X-ray film densitometry have been described (Stumm et al., 2002; Abe et al., 2014). Axon outgrowth from thalamus explants was quantified by measuring the 20 longest axons in each explant with ImageJ using the NeuronJ plug-in (illustrated in Figure 8A). In each experimental run, axon length was normalized to control mean of the respective run. Statistics were calculated and plotted with GraphPad Prism and IBM SPSS software. Adobe Photoshop and InDesign CS6 were used to process and arrange figures.

RESULTS

Meninges are the primary CXCL12 source for early CXCR4-expressing thalamic neurons

Little is known about the expression of the *Cxcl12*, *Cxcr4* and *Cxcr7* genes in the developing thalamus. We thus analyzed spatiotemporal expression profiles of these genes by highly sensitive *in situ* hybridization with radiolabeled probes, focusing on the *Gbx2*$^{+}$ domain in the developing diencephalon. This domain corresponds to the mantle zone of the thalamus (tMZ) and is composed of postmitotic glutamatergic neurons, which project to the cortex and constitute the nuclear complex that is traditionally viewed as the thalamus (Chatterjee and Li, 2012). First, we examined E12.5, E13.5 and E14.5 embryos (Figure 1A-O), because most thalamic neurons are generated during this period (Suzuki-Hirano et al., 2011). Adjacent sections revealed strong expression of *Cxcr4* and *Cxcr7* in the progenitor domain along the 3rd ventricle at E12.5 and E.13.5. In contrast, the tMZ expressed *Cxcr4* but not *Cxcr7* at this early stage of thalamic development (Figure 1A,C,E,F,H,J). At E14.5, the lateral tMZ still exhibited contiguous strong *Cxcr4* signal and virtually no *Cxcr7* signal while the medial tMZ contained scattered *Cxcr4*$^{+}$ cells and uniform faint *Cxcr7* signal (Figure 1K,M,O). *Cxcl12* was expressed in the meninges covering the thalamus but, apart from some forming blood vessels, was not detected within the thalamus during E12.5 - E14.5 (Figure 1B,G,L).

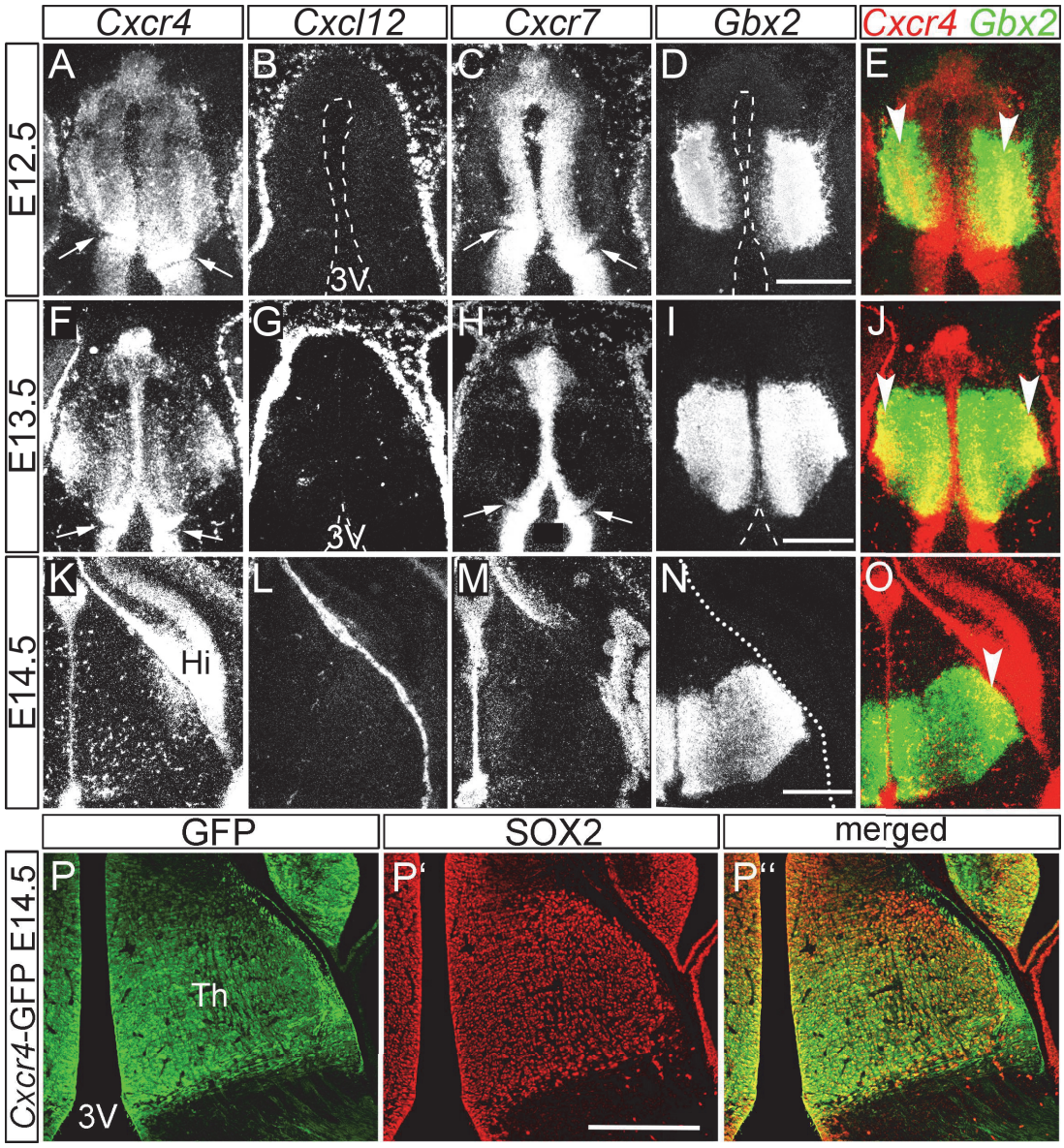


Figure 1. Patterns of *Cxcr4, Cxcr7* and *Cxcl12* in the early thalamus

A-O, Darkfield micrographs of emulsion-dipped coronal sections show the thalamus after *in situ* hybridization with ^{35}S-labeled probes for *Cxcr4* (A,F,K), *Cxcl12* (B,G,L), *Cxcr7* (C,H,M) and *Gbx2* (D,I,N) at E12.5 (A-E), E13.5 (F-J) and E14.5 (K-O). *E,J* and *O* are overlays of false color displays of the adjacent sections shown in *A/D*, *F/I* and *K/N*.

A,E,F,J,K,O *Cxcr4* is expressed in the progenitor domain along the midline and in the *Gbx2*$^+$ differentiation area of the dorsal thalamus. Arrowheads in *E,J* and *O* point to *Cxcr4*-expressing lateral thalamic nuclei.

B,G,L, *Cxcl12* is present in the meninges covering the thalamus.

C,H,M, *Cxcr7* is expressed in the progenitor domain along the midline.

A,C,F,H Arrows point to the *zona limitans intrathalamica* for orientation.

P-P´´, Confocal images show immunostained GFP and SOX2 in a coronal section of an E14.5 *Cxcr4*-GFP reporter mouse.

Abbreviations: 3V, third ventricle; Th, thalamus. **Scale bars:** 500 µm in *D,I,N,P´*.

We confirmed localization of *Cxcr4* in the dorsal thalamus by dual labeling with the thalamic marker SOX2 (Vue et al., 2007). Specifically, dual immunofluorescence for GFP and SOX2 showed that the entire SOX2$^+$ domain was *Cxcr4*-GFP$^+$ at E14.5 (Figure 1P-P´´). Because of its slow turnover, GFP traces back brain structures that expressed the reporter during the

preceding days. Thus, the *Cxcr4*-GFP pattern at E14.5 corresponds to the finding that virtually the entire forming thalamus expresses *Cxcr4* mRNA at E12.5 and E13.5. Dual *in situ* hybridization showed that at E14.5 *Cxcr4* mRNA was still expressed in *Sox2*$^{+}$ and *Gbx2*$^{+}$ parts of dorsal thalamus (Figure 2A-D).

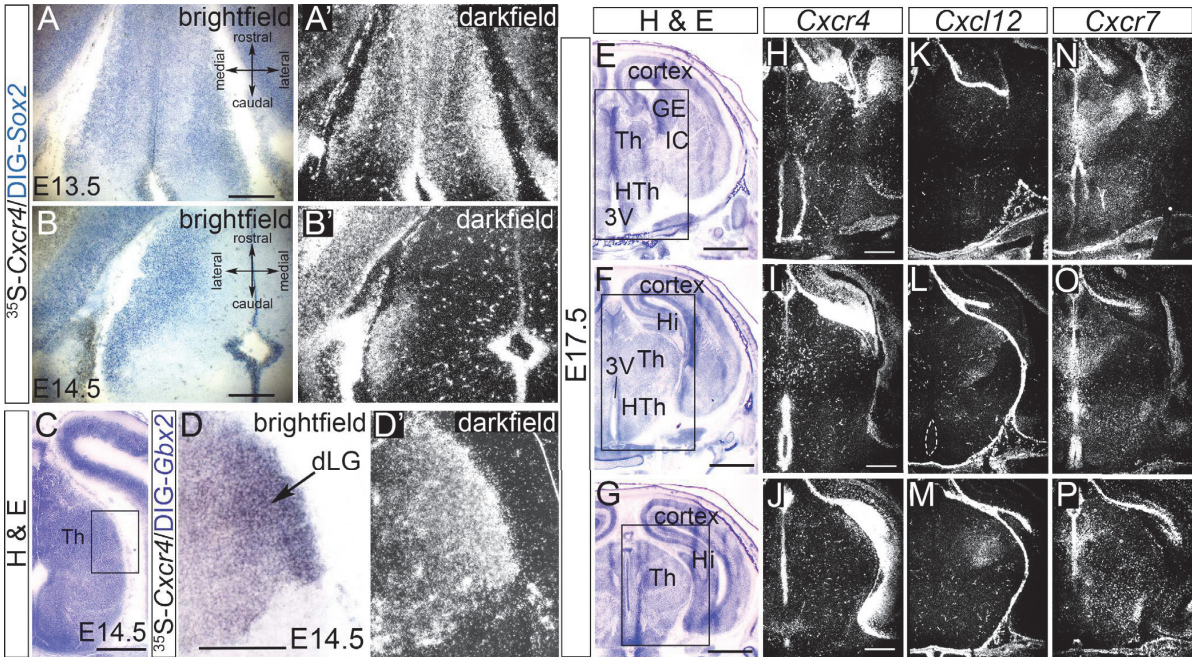


Figure 2. Transient *Cxcr4* expression in the thalamic mantle zone.
A,B,D, Dual *in situ* hybridization for *Cxcr4*/*Sox2* (A,B) and *Cxcr4*/*Gbx2* (D) at E13.5 (A) and E14.5 (B,D). *Cxcr4* was detected with a ^{35}S-labeled probe. *Sox2* and *Gbx2* were detected with digoxigenin (DIG)-labeled probes. *A,B,D* are brightfield views and *A´,B´,D´* are darkfield views of the same specimens. *A,B* are horizontal and *D* is a coronal (D) section. *Cxcr4* signal is present in the *Sox2*$^{+}$ and *Gbx2*$^{+}$ thalamic mantle zone.
C, The H&E-stained coronal section shows the diencephalon, the box corresponds to the thalamic area shown in *D*.
E-G, Boxes in the H&E-stained sections correspond to the thalamic area shown in *H-P*. Images show the thalamus at rostral (upper panel), mid (middle panel) and caudal levels (lower panel).
H-P, Darkfield micrographs of emulsion-dipped coronal sections after *in situ* hybridization with ^{35}S-labeled probes for *Cxcr4* (H-J), *Cxcl12* (K-M) and *Cxcr7* (N-P).
Abbreviations: 3V, third ventricle; IC; internal capsule; dLG, dorsal lateral geniculate; GE, ganglionic eminence; Hi, hippocampus; HTh, hypothalamus; Th, thalamus. **Scale bars:** 200 µm in *A,B,D* and 500 µm in *C,H-J* and 1 mm in *E-G*.

Next, we examined stages during which thalamic nuclei begin to differentiate (E16.5, E17.5, P0; demonstrated for E17.5 in Figure 2E-P). We observed that *Cxcr4* was gradually down-regulated while *Cxcl12* and *Cxcr7* were up-regulated in the thalamus. Moderate to faint contiguous *Cxcr4* signal could be detected until E17.5 in dorsolateral nuclei. Scattered, strongly *Cxcr4*$^{+}$ cells were present throughout the thalamus at all stages.

Our gene expression analyses suggest that CXCR4 is the predominantly expressed CXCL12 receptor in early postmitotic thalamocortical neurons and that the meninges are the

primary diencephalic CXCL12 source. To test this assumption, we studied CXCR4 receptor activation, making use of UMB-2 and 2B11 anti-CXCR4 antibodies. UMB-2 recognizes its epitope within the amino acid residues 343-52 only when the serine cluster S346-8 is unphosphorylated. As this cluster becomes immediately phosphorylated upon CXCL12 stimulation (Mueller et al., 2013), UMB-2 binds only inactive CXCR4 receptors (Figure 3A,B,D,E: $CXCR4^{inact}$). 2B11 is directed against the CXCR4 N terminus and recognizes CXCR4 receptors independently of their activation state (Figure 3C: CXCR4). By applying these antibodies to adjacent sections from E13.5 *Cxcr4*-GFP animals, we found that 2B11 and UMB-2 generated staining medially in the *Cxcr4*-GFP^+ thalamus while only 2B11 produced staining laterally in the *Cxcr4*-GFP^+ thalamus (Figure 3A-C, asterisks identify the lateral thalamus). To prove that lack in UMB-2 signal reflects CXCR4 activation in the lateral thalamus, we compared thalamic UMB-2 staining in E14.5 *Cxcl12*$^{-/-}$ and control littermates. In the absence of CXCL12, there was a profound increase in UMB-2 signal in the lateral thalamus (Figure 3D,E, asterisks). These findings indicate active signaling of the CXCL12/CXCR4 pathway in early thalamic neurons.

Development of the thalamus in *Cxcl12*$^{-/-}$ mice

We then asked if CXCL12 is required for thalamus formation. We thus examined thalamic size and shape in *Cxcl12*$^{-/-}$ mice at different developmental stages. At E14.5, we alternately hybridized 5 sections at different rostrocaudal planes for *Gbx2* and *Sox2* to identify the boundaries between thalamus and epithalamus (*Gbx2*) and thalamus and prethalamus (*Sox2*) (Vue et al., 2007). The thalamic area was defined as shown in the insets in Figure 3H. We found that size and shape of the *Gbx2* and *Sox2* expression domains were not altered in *Cxcl12*$^{-/-}$ mice (Figure 3F-H). The thalamic boundaries were sharply delineated in the mutants and appeared similar to controls (shown for *Sox2* in Figure 3F,G). We then immunostained for SOX2 at E16.5 (Figure 3I-K) and hybridized for vesicular glutamate transporter 2 (*Vglut2/ Slc17a6*; Figure 3L-N) at E18.5. Again, we found the labeled areas to be of normal size and shape in *Cxcl12*$^{-/-}$ embryos, suggesting that the thalamus forms independently of CXCL12. However, since *Gbx2*, *Sox2* and *Vglut2* do not resolve thalamic subnuclei, these findings do not preclude the possibility that genesis of distinct thalamic nuclei might require CXCL12.

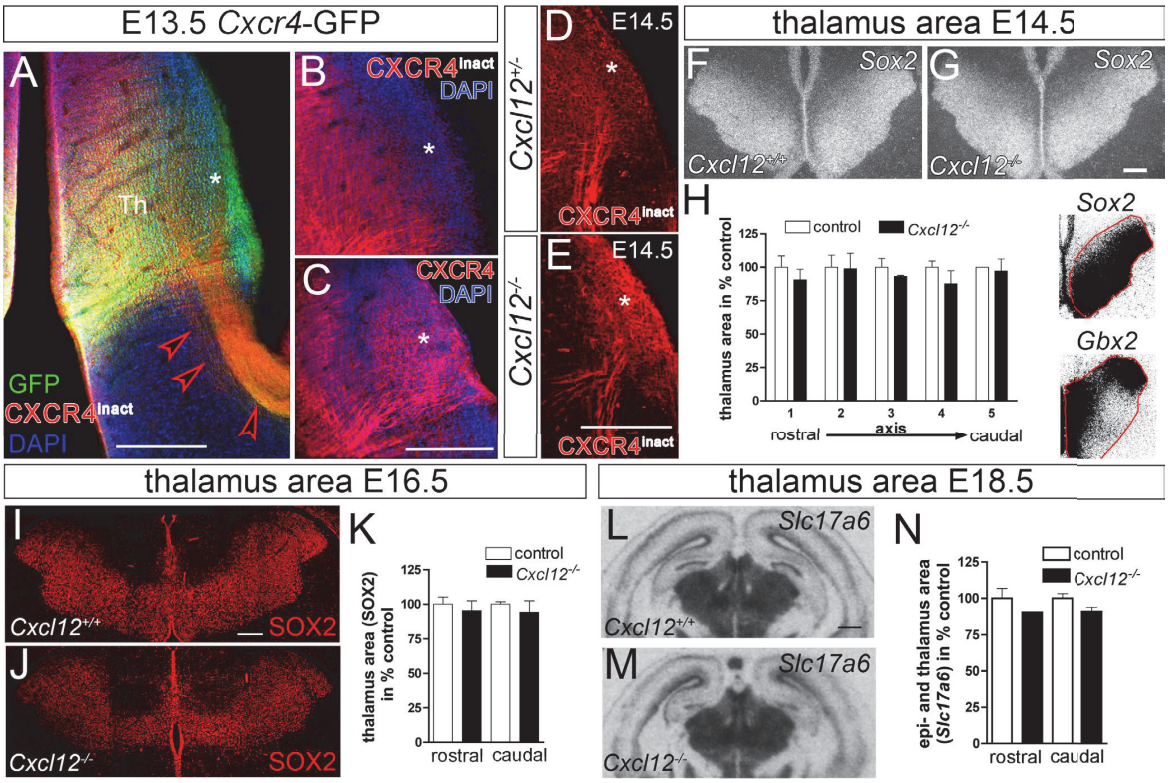


Figure 3. CXCL12 activates CXCR4 receptors in the developing thalamus but is dispensable for thalamus formation.

A-E, Confocal images show immunohistochemistry for inactive/non-phosphorylated CXCR4 ($CXCR4^{inact}$; A,B,D,E) and total CXCR4 (CXCR4; C) in coronal thalamus sections.

A, Overlay of immunostained GFP, $CXCR4^{inact}$ and DAPI in an E13.5 *Cxcr4*-GFP reporter mouse shows CXCR4 in the thalamocortical projection (arrowheads). Note that the lateral thalamus (asterisk) contains only sparse $CXCR4^{inact}$ signal despite being *Cxcr4*-GFP^{+}.

B,C, The lateral thalamus (asterisk) shows scant signal for $CXCR4^{inact}$ (B) but strong signal for CXCR4 (C). Sections were counterstained with DAPI.

D,E, Immunohistochemistry for $CXCR4^{inact}$ in an E14.5 *Cxcl12*$^{+/-}$ (control; D) and a *Cxcl12*$^{-/-}$littermate (E) demonstrates that $CXCR4^{inact}$ increases in the lateral thalamus (asterisks) in the absence of CXCL12.

F,G, Darkfield micrographs of emulsion-dipped coronal sections through the thalamus of an E14.5 *Cxcl12*$^{+/+}$ and a *Cxcl12*$^{-/-}$ littermate after *in situ* hybridization with a ^{35}S-labeled probe for *Sox2*.

H, Matching thalamus sections, cut at 5 rostrocaudal sectional levels in E14.5 control (n=3) and *Cxcl12*$^{-/-}$ littermates (n=3), were hybridized for *Gbx2* (sectional levels 1,3 and 5) and *Sox2* (sectional levels 2 and 4) as thalamus markers. The marker^{+} area was determined in micrographs of emulsion-dipped sections after setting a threshold as shown in the insets to the right (signal above threshold appears black).

I, Confocal images show SOX2-immunoreactivity in the thalamus of an E16.5 *Cxcl12*$^{+/+}$ (I) and a *Cxcl12*$^{-/-}$ littermate (J).

K, The thalamus area was determined based on SOX2-immunoreactivity at a rostral and at a caudal sectional level in E16.5 control and *Cxcl12*$^{-/-}$ littermates (n=5 each).

L,M, X-ray autoradiograms show *Slc17a6* (*Vglut2*) mRNA in hybridized coronal head sections of an E18.5 *Cxcl12*$^{+/+}$ (L) and a *Cxcl12*$^{-/-}$ littermate (M).

N, The *Slc17a6* mRNA-positive area corresponding to thalamus and epithalamus was determined in X-ray autoradiograms at a rostral and at a caudal sectional level in E18.5 *Cxcl12*$^{+/+}$ and *Cxcl12*$^{-/-}$ littermates (n=4 each).

H,K,N, The thalamus area is not altered in *Cxcl12*$^{-/-}$ mice (data are mean+SEM).

Scale bars: 200 µm in *A,C,E,G,I* and 500 µm in *L*.

CXCR4$^+$ TCAs traverse a *Cxcl12*-expressing domain in the cortical SVZ

Having identified CXCR4 in neurons forming thalamocortical projections, we wondered if CXCR4 is functionally involved in TCA development. To address this question, we employed the *Cxcr4*-GFP reporter for visualization of TCAs emerging from *Cxcr4*-expressing neurons. Co-immunostaining for GFP and the axonal marker L1 revealed *Cxcr4*-GFP/L1$^+$ axons originating in the thalamus and extending through the internal capsule toward the cortex (Figure 4A-B´´). GFP patterns in the E14.5 cortex (Figure 4B-B´´) were consistent with presence of *Cxcr4*-GFP in axons, interneurons and Cajal-Retzius cells and absence of *Cxcr4*-GFP from cortical PNs (Stumm et al., 2003). These findings establish that *Cxcr4*-GFP specifically labels TCAs and rule out the possibility that corticothalamic axons are labeled.

Next, we examined where along the TCA trajectory *Cxcl12* is expressed during E12.5 – E14.5 by comparing patterns of *Cxcr4*-GFP and *Cxcl12* mRNA. Areas in the ventral thalamus, prethalamus, internal capsule and ventral telencephalon were traversed by *Cxcr4*-GFP$^+$ TCAs before E13.5 but exhibited only very sparse *Cxcl12* signal (shown for E13.5 in Figure 4C,D). By E13.5, *Cxcr4*-GFP$^+$ TCAs reached the pallial-subpallial boundary and started to enter the *Cxcl12*-expressing domain in the cortical SVZ (Figure 4C,D,F). Between E13.5 and E14.5, *Cxcr4*-GFP$^+$ TCAs crossed the *Cxcl12*-expressing SVZ and progressed considerably within the cortex, covering more than half of its lateromedial extent (Figure 4A-F).

A prerequisite for a direct influence of CXCL12 on TCAs is the presence of CXCR4 receptors in the axonal compartment. We observed prominent CXCR4 immunolabeling in *Cxcr4*-GFP$^+$ TCAs entering the internal capsule (Figure 3A, arrowheads). The receptor could also be identified in the distal axonal compartment (not shown) but the signal was less intense than proximally, where TCAs are bundled. To confirm that CXCR4 is targeted toward the growth cone of neurites extending from thalamic neurons, we immunostained CXCR4 and SOX2 in thalamic cultures. This revealed CXCR4 presence at the soma, neurite and growth cone of SOX2$^+$ thalamic neurons (Figure 4G-G´´). Taken together, these findings prompt to suggest that the thalamic meninges and the cortical SVZ are CXCL12 sources that influence TCA development through somatic and axonal CXCR4 stimulation, respectively.

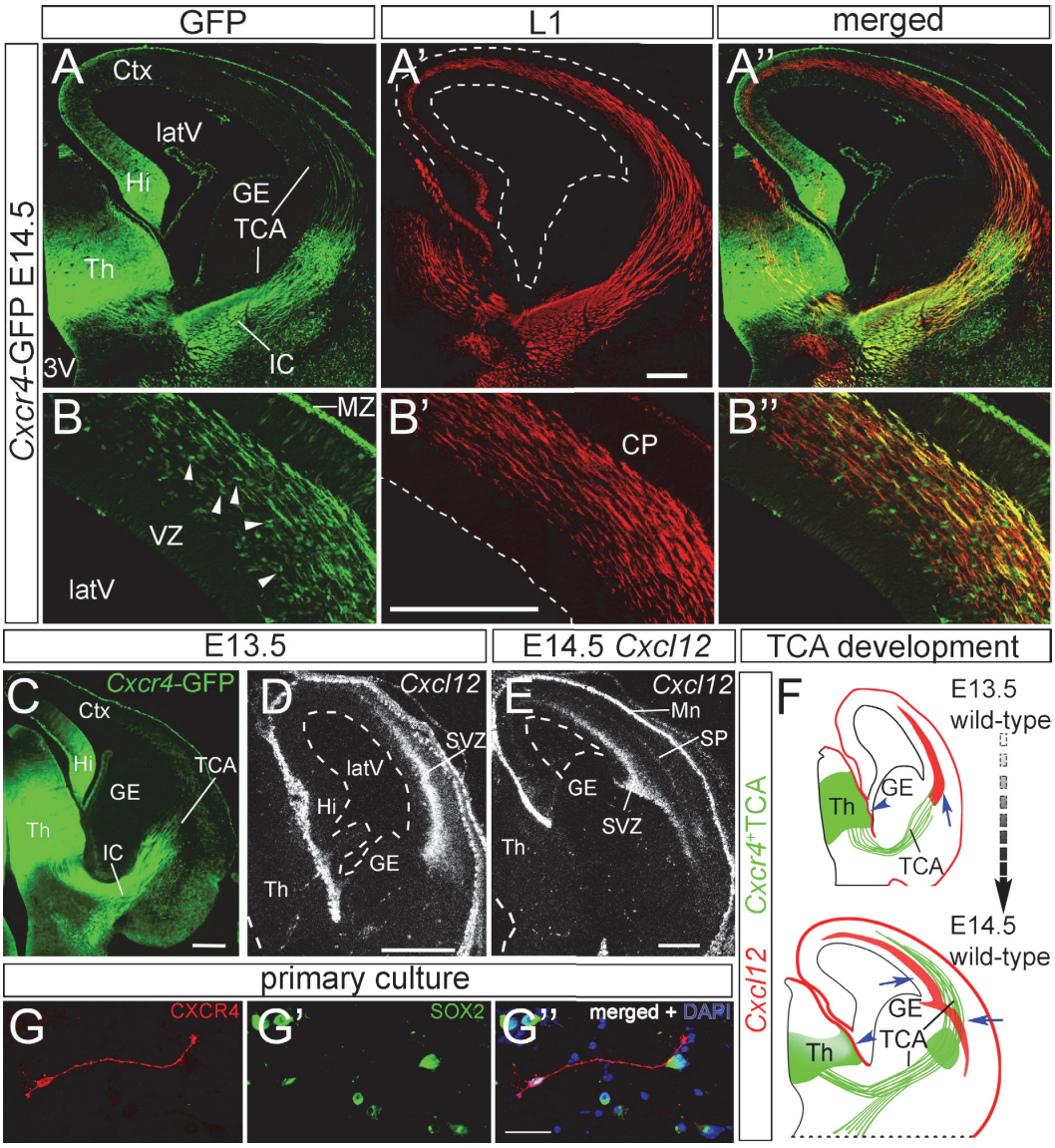


Figure 4. *Cxcr4*-GFP identifies TCAs

A-B´´, Confocal images show immunostained GFP and the axonal marker L1 in an E14.5 *Cxcr4*-GFP reporter mouse. *Cxcr4*-GFP labels thalamocortical axons (TCA) identified by L1. Presumptive GFP$^+$ interneurons (arrowheads in B) are L1-negative.

C, Confocal image of a *Cxcr4*-GFP reporter mouse shows that GFP$^+$ TCAs have traversed the internal capsule (IC) and reached the SVZ at E13.5.

D,E, Darkfield micrographs of emulsion-dipped coronal sections at E13.5 (D) and E14.5 (E) after *in situ* hybridization with a ^{35}S-labeled probe for *Cxcl12*. *Cxcl12* is expressed in the cortical subventricular zone (SVZ), subplate (SP) and meninges covering thalamus and cortex (Mn).

F, Schematic summarizing patterns of *Cxcr4*-GFP and *Cxcl12* mRNA at E13.5 and E14.5. Arrows indicate putative cortical and arrowheads putative meningeal stimulation sites of *Cxcr4*$^+$ TCAs and thalamic neurons, respectively.

G, Dissociated thalamic neurons were immunostained for SOX2 and CXCR4 and counterstained with DAPI. Note CXCR4 in the neurite and the growth-cone.

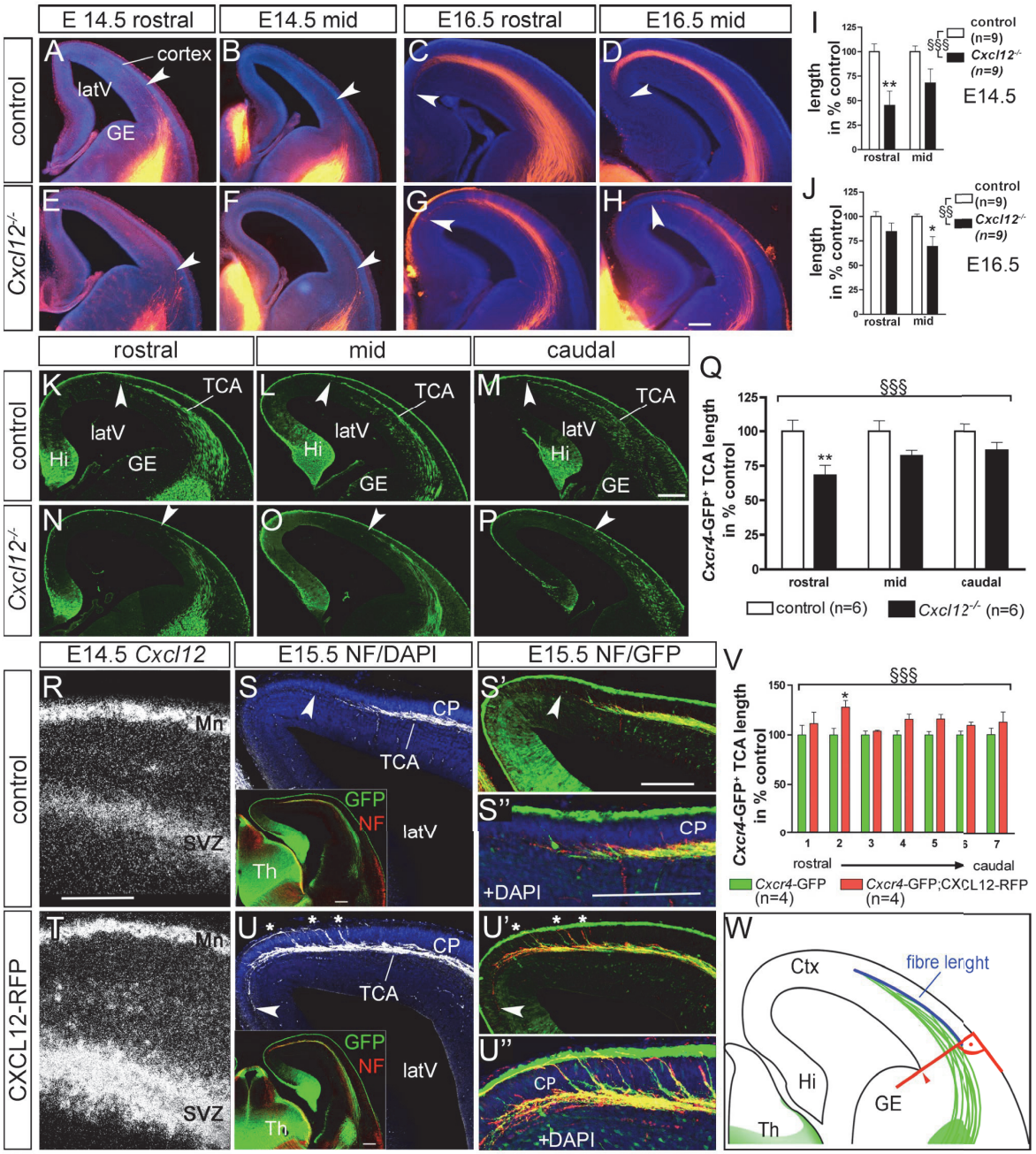
Abbreviations: 3V, third ventricle; Ctx, cortex; CP, cortical plate; GE, ganglionic eminence; Hi, hippocampal anlage; latV, lateral ventricle; Th, thalamus; VZ, ventricular zone. **Scale bars:** 200 µm in *A',B',C-E* and 20 µm in *G*.

CXCL12 promotes intracortical TCA elongation

To directly test if CXCL12 is involved in TCA development, we traced the trajectories by DiI placement in control and *Cxcl12*$^{-/-}$ E14.5 and E16.5 thalami (Figure 5A-H). No misrouting of DiI-labeled TCAs was observed in the ventral thalamus, internal capsule and ventral telencephalon of *Cxcl12*$^{-/-}$ mice. However, labeled axons appeared less developed in the cortex of these mutants. Measurement of the intracortical length of DiI-labeled TCAs confirmed significantly delayed TCA growth in *Cxcl12*$^{-/-}$ embryos as compared to control littermates (E14.5, $p=0.0003$ and E16.5, $p=0.009$ for genotype, 2way-ANOVA, Figure 5I,J). Length reductions by 55.0% at the rostral sectional level at E14.5 ($p<0.01$) and by 31.0% at the mid sectional level at E16.5 ($p<0.05$) were significant in the post-hoc test.

To focus specifically on *Cxcr4*$^{+}$ TCAs, we crossed *Cxcr4*-GFP into the *Cxcl12*$^{+/-}$ mouse line and generated *Cxcr4*-GFP;*Cxcl12*$^{-/-}$ mutant embryos and *Cxcr4*-GFP control littermates (Figure 5K-P). Length measurement of GFP^{+} fibers in the E14.5 cortex at three rostro-caudal sectional levels showed an overall growth reduction in the mutants as compared to control littermates ($p=0.0004$ for genotype, 2way-ANOVA, Figure 5Q). Length reduction by 31.8% at the rostral sectional level reached significance in the post-hoc test ($p<0.01$). The delayed growth was not accompanied by misrouting of *Cxcr4*-GFP^{+} TCAs in *Cxcr4*-GFP;*Cxcl12*$^{-/-}$ embryos.

Next, we tested if CXCL12 overexpression accelerates TCA development. We thus generated double-transgenic mice expressing *Cxcr4*-GFP and a CXCL12-RFP fusion protein under control of the *Cxcl12* promoter (CXCL12-RFP). In CXCL12-RFP mice, additional *Cxcl12* gene copies result in increased *Cxcl12* mRNA expression (Figure 5R,T) and, hence, in an increase of functional CXCL12. Immunofluorescent co-labeling of *Cxcr4*-GFP and Neurofilamant (NF) as a TCA marker in E15.5 *Cxcr4*-GFP control and CXCL12-RFP;*Cxcr4*-GFP double-transgenic mice revealed accelerated TCA growth following CXCL12 overexpression (Figure 5S,U,V). Remarkably, in an area corresponding to the visual cortex of CXCL12-RFP embryos, TCAs entered the cortical plate prematurely and even targeted the marginal zone (asterisks in Figure 5U). Measurement of intracortical TCA length at seven rostro-caudal sectional planes confirmed increased TCA length in CXCL12-RFP animals ($p=0.0001$ for genotype, 2way-ANOVA, Figure 5V). An increase in intracortical TCA length by 28% in the second sectional plane was significant in the post-hoc test ($p<0.05$). These gain and loss of function experiments establish that CXCL12 promotes intracortical progression of TCAs.


E 14.5 rostral
E14.5 mid
E16.5 rostral
E16.5 mid
control
Cxcl12-/-
cortex
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control (n=9)
Cxcl12-/- (n=9)
E14.5
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length in % control
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caudal
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E14.5 Cxcl12
E15.5 NF/DAPI
E15.5 NF/GFP
CXCL12-RFP
Mn
SVZ
CP
GFP
NF
Th
+DAPI
V
Cxcr4-GFP
(n=4)
Cxcr4-GFP;CXCL12-RFP
(n=4)
W
Ctx
fibre lenght

Figure 5. CXCL12 promotes intracortical TCA elongation
A-H, Thalamic DiI labeling at E14.5 (A,B,E,F) and E16.5 (C,D,G,H) shown in coronal sections at a rostral and a mid sectional level in control (A-D) and *Cxcl12*$^{-/-}$ embryos (E-H). Arrowheads point to positions of the most advanced DiI-labeled TCAs.
I,J, Intracortical length of DiI-labeled TCAs in E14.5 (I) and E16.5 (J) control embryos and *Cxcl12*$^{-/-}$ littermates at a rostral and a mid sectional level.
K-P, Confocal images show immunostained GFP in control (K-M) and *Cxcl12*$^{-/-}$ (N-P) E14.5 *Cxcr4*-GFP reporter mice at rostral (K,N), mid (L,O) and caudal sectional planes (M,P). Arrowheads point to positions of the most advanced GFP^{+} TCAs.
Q, Intracortical length of *Cxcr4*-GFP^{+} TCAs in control and *Cxcl12*$^{-/-}$ E14.5 *Cxcr4*-GFP reporter mice at rostral, mid and caudal sectional planes.
R,T, Darkfield views of the cerebral cortex at E14.5 after *in situ* hybridization with a ^{35}S-labeled probe for *Cxcl12*. Comparison of a control (R) and a CXCL12-transgenic littermate overexpressing CXCL12 under the *Cxcl12* promoter (T) reveals increased *Cxcl12* mRNA expression in the transgenic animal.
S,U, Confocal images show a *Cxcr4*-GFP single-transgenic control (S) and a *Cxcr4*-GFP;CXCL12-RFP double-transgenic littermate at E15.5 (U). Immunostained Neurofilament (NF) is white in *S* and *U* and red in *S′,S′′,U′* and *U′′*. GFP is green in *S′,S′′,U′* and *U′′*. DAPI is shown in blue in *S,U,S′′* and *U′′*. Arrowheads point to the location of the most advanced stained TCAs; asterisks point to TCAs crossing the cortical plate prematurely (U,U').
V, Intracortical length of *Cxcr4*-GFP^{+} TCAs in *Cxcr4*-GFP single transgenic and *Cxcr4*-GFP;CXCL12-RFP double-transgenic E15.5 mice in seven sectional planes along the rostro-caudal axis.
W, Scheme illustrating measurement of intracortical TCA length in *I,J,Q,V*.
Statistics in I,J,Q,V: $^{§§§}p<0.001$, $^{§§}p<0.01$ for genotype (2way-ANOVA). $^{**}p<0.01$, $^{*}p<0.05$ (Bonferroni's post-hoc test). Numbers of analyzed embryos are indicated.
Abbreviations: Ctx, cortex; CP, cortical plate; GE, ganglionic eminence; Hi, hippocampal anlage; latV, lateral ventricle; Mn, meninges; SVZ, subventricular zone; TCA, thalamocortical afferents; Th, thalamus.
Scale bars: 200 µm in *H,M,R,S-S′′*.

IPC-derived CXCL12 ensures efficient TCA growth and interneuron dispersion

We then asked, if SVZ-derived CXCL12 is sufficient to promote TCA growth. To address this, we deleted *Cxcl12* selectively in IPCs using *Tbr2*$^{Cre/+}$-mediated excision of the CXCL12-encoding exon2 in *Cxcl12*$^{LoxP/-}$ mice (*Cxcl12cKO*$^{Tbr2\text{-}Cre}$) (Costello et al., 2011; Tzeng et al., 2011). *In situ* hybridization with an exon2-selective probe confirmed that *Cxcl12* expression was indeed disrupted in the SVZ and intact in the meninges in E14.5 *Cxcl12cKO*$^{Tbr2\text{-}Cre}$ mice (Figure 6A,B). To visualize *Cxcr4*-expressing TCAs in these mutants, we generated E14.5 *Cxcr4*-GFP;*Cxcl12cKO*$^{Tbr2\text{-}Cre}$ mice by crossbreeding *Tbr2*^{Cre} driver, *Cxcr4*-GFP reporter and *Cxcl12*$^{LoxP/-}$ mice (Figure 6C,D). Intracortical length of *Cxcr4*-GFP^{+} TCAs, determined at three rostro-caudal sectional levels, was reduced in the conditional mutants as compared to littermate controls ($p<0.0001$ for genotype, 2way-ANOVA, Figure 6E). The post-hoc test identified that TCAs were significantly shorter by 28%, 21% and 25% at the rostral ($p<0.001$), medial ($p<0.05$) and caudal ($p<0.01$) level in the conditional mutants.

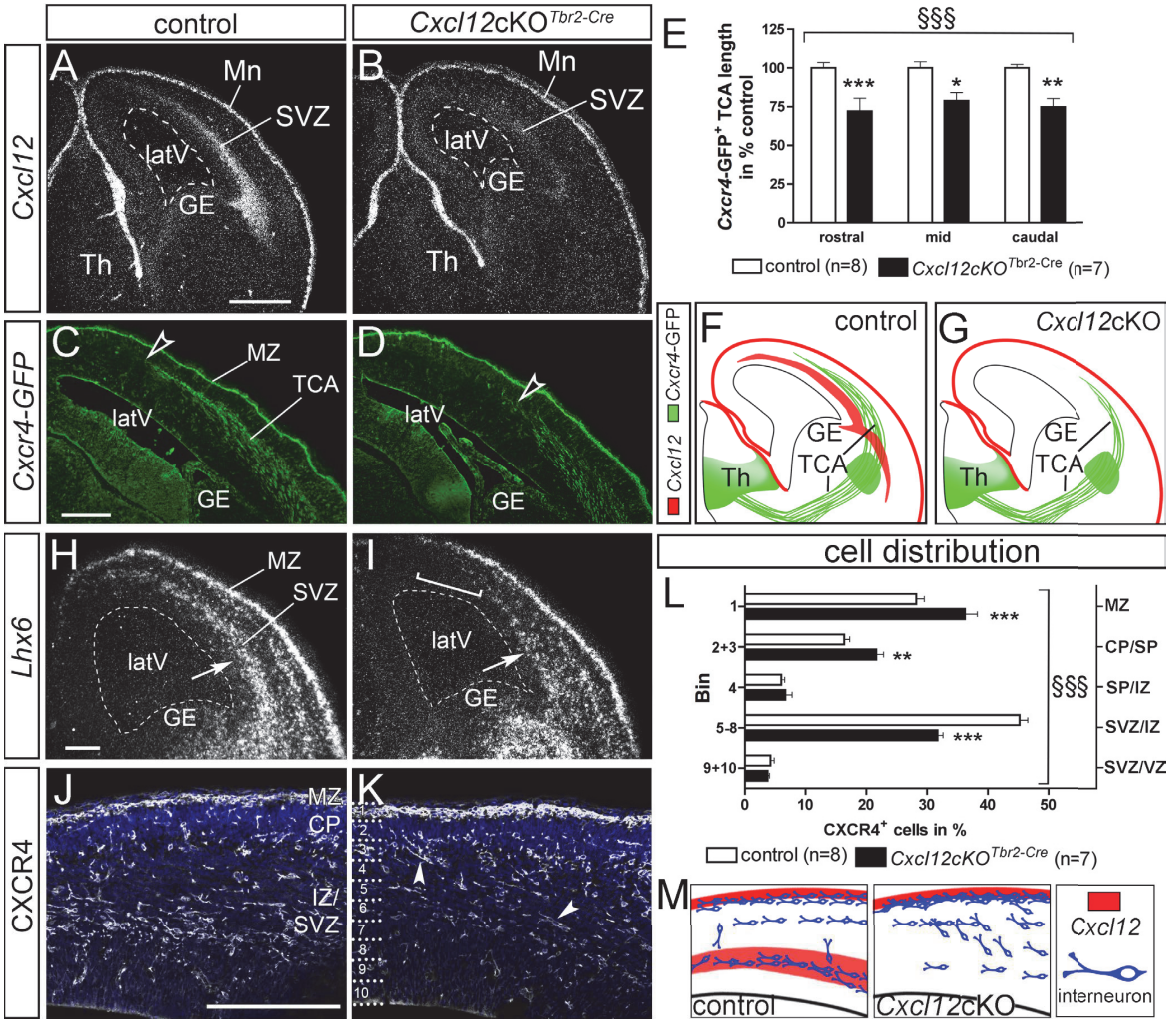

Figure 6. IPC-derived CXCL12 ensures efficient intracortical TCA growth and interneuron dispersion.
A,B, Darkfield micrographs of emulsion-dipped E14.5 coronal brain sections after *in situ* hybridization with a ^{35}S-labeled probe corresponding to exon 2 of the *Cxcl12* gene (exon2 is loxP-flanked in *Cxcl12*^{LoxP} mice). *Cxcl12* is expressed in the cortical subventricular zone (SVZ) of a *Tbr2*$^{Cre/+}$;*Cxcl12*$^{+/+}$ (control, A) but not in the SVZ of a *Tbr2*$^{Cre/+}$;*Cxcl12*$^{LoxP/-}$ mouse (*Cxcl12*cKO, B). *Cxcl12* expression is intact in the meninges (Mn) of the *Cxcl12*cKO.
C,D, Confocal images of immunostained GFP in the cortex at mid rostrocaudal sectional level in E14.5 mice carrying the *Cxcr4*-GFP reporter. Arrowheads pointing to the most advanced GFP$^+$ TCAs demonstrate that GFP$^+$ TCAs are more advanced in the control (C) than in the *Cxcl12*cKO (D).
E, Intracortical length of *Cxcr4*-GFP$^+$ TCAs in control (n=8) and *Cxcl12*cKO embryos (n=7).
F,G, Schemes illustrating the TCA phenotype in the *Cxcl12*cKO.
H,I, Darkfield micrographs of emulsion-dipped coronal sections through the E14.5 cortex after *in situ* hybridization with a ^{35}S-labeled probe for *Lhx6*. Fewer *Lhx6*$^+$ interneurons populate the SVZ (arrows) in the *Cxcl12*cKO (I) than in the control (H). Note *Lhx6*$^+$ interneurons are absent in the dorsomedial SVZ in the *Cxcl12*cKO (bracket in I).
J,K, Confocal images of immunostained CXCR4 (white) and DAPI (blue) in coronal cortical sections of E14.5 control (J) and *Cxcl12cKO* mice (K). Cortical bins are shown in *K*. There are fewer CXCR4$^+$ interneurons in the SVZ (bins 5 - 8) and more CXCR4$^+$ interneurons in upper cortical layers (bins 1 - 3) of the *Cxcl12cKO* (K) than in the corresponding areas of the control (J). More CXCR4$^+$ interneurons exhibit oblique orientation in the *Cxcl12*cKO (arrowheads in K) than in the control, indicating abnormal, surface-directed migration of these cells.
L, Distribution of CXCR4$^+$ cells in the cortex of E14.5 control (n=8) and Cxcl12*cKO* mice (n=7). Data are mean+SEM of CXCR4$^+$ cells per area in percent of all CXCR4$^+$ cells.
M, Scheme illustrating the interneuron phenotype in the *Cxcl12*cKO.
Statistics: §§§p<0.0001 for bin/genotype interaction (2way-ANOVA). ***p<0.001, **p<0.01, *p<0.05, (Bonferroni's post-hoc test compared to control); n numbers as indicated. **Abbreviations:** CP, cortical plate; GE, ganglionic eminence; IZ, intermediate zone; latV, lateral ventricle; Mn, meninges; MZ, marginal zone; SP, subplate; SVZ, subventricular zone; Th, thalamus; VZ, ventricular zone. **Scale bars:** 500 µm in *A* and 200 µm in *C,H,J*.

Having shown that IPC-derived CXCL12 promotes elongation of TCAs in the SVZ, we wondered if IPCs have a similar effect on interneurons. It is well established that the CXCL12/CXCR4/CXCR7 signaling module regulates interneuron migration in the cortex, but the respective contributions of meningeal- and IPC-derived CXCL12 to this process has not dissected by targeting the *Cxcl12* gene selectively in one of these tissues. We thus examined interneuron positioning in *Cxcl12cKO*$^{Tbr2\text{-}Cre}$ mice. As GFP^{+} TCAs mask the position of weakly GFP-stained interneurons in the cortex of *Cxcr4*-GFP mice, we hybridized for *Lhx6,* a marker of medial ganglionic eminence-derived interneurons. In the two examined E14.5 *Cxcl12cKO*$^{Tbr2\text{-}Cre}$ mice, the lateral SVZ contained fewer *Lhx6*$^{+}$ cells than the corresponding region in control littermates (arrows in Figure 6H,I,). The medial SVZ of the conditional mutants was devoid of *Lhx6*$^{+}$ cells while it was readily populated by *Lhx6*$^{+}$ cells in the controls (brackets in Figure 6H,I). To confirm these observations, we immunostained cortices of E14.5 *Cxcl12cKO*$^{Tbr2\text{-}Cre}$ mice for CXCR4 and counted stained cells in ten cortical bins in a mid cortical area (Figure 6J-L). In *Cxcl12cKO*$^{Tbr2\text{-}Cre}$ mice, there was a significant reduction in CXCR4^{+} cells in bins 5 - 8 corresponding to the SVZ/IZ ($p<0.001$) and a significant increase in CXCR4^{+} cells in bin 1 ($p<0.001$) and bins 2/3 ($p<0.01$) corresponding to the MZ and CP, respectively (2way-ANOVA and post-hoc test). Leading processes of CXCR4-immunoreactive interneurons migrating in the SVZ/IZ in *Cxcl12cKO*$^{Tbr2\text{-}Cre}$ mice often exhibited an oblique orientation (Figure 6K, arrowheads), suggesting that these cells were heading toward the MZ. In contrast, CXCR4-immunoreactive interneurons migrating in the SVZ/IZ of control mice had a more tangential orientation. In conclusion, CXCL12 produced by IPCs ensures efficient TCAs growth and interneuron dispersion in the SVZ/IZ (Figure 6F,G,M).

CXCL12/CXCR4 and Slit/Robo pathways interact to regulate TCA growth

We then returned to TCA development, asking how CXCL12 mediates its growth-promoting effect on this system. Mechanisms controlling the growth rate of axons are still poorly understood. Recently, regulation of *Robo1* transcription in thalamocortical neurons was shown to mediate a switch in the TCA growth rate with low *Robo1* levels being associated with fast progression of the axons before E14.5 (Mire et al., 2012).

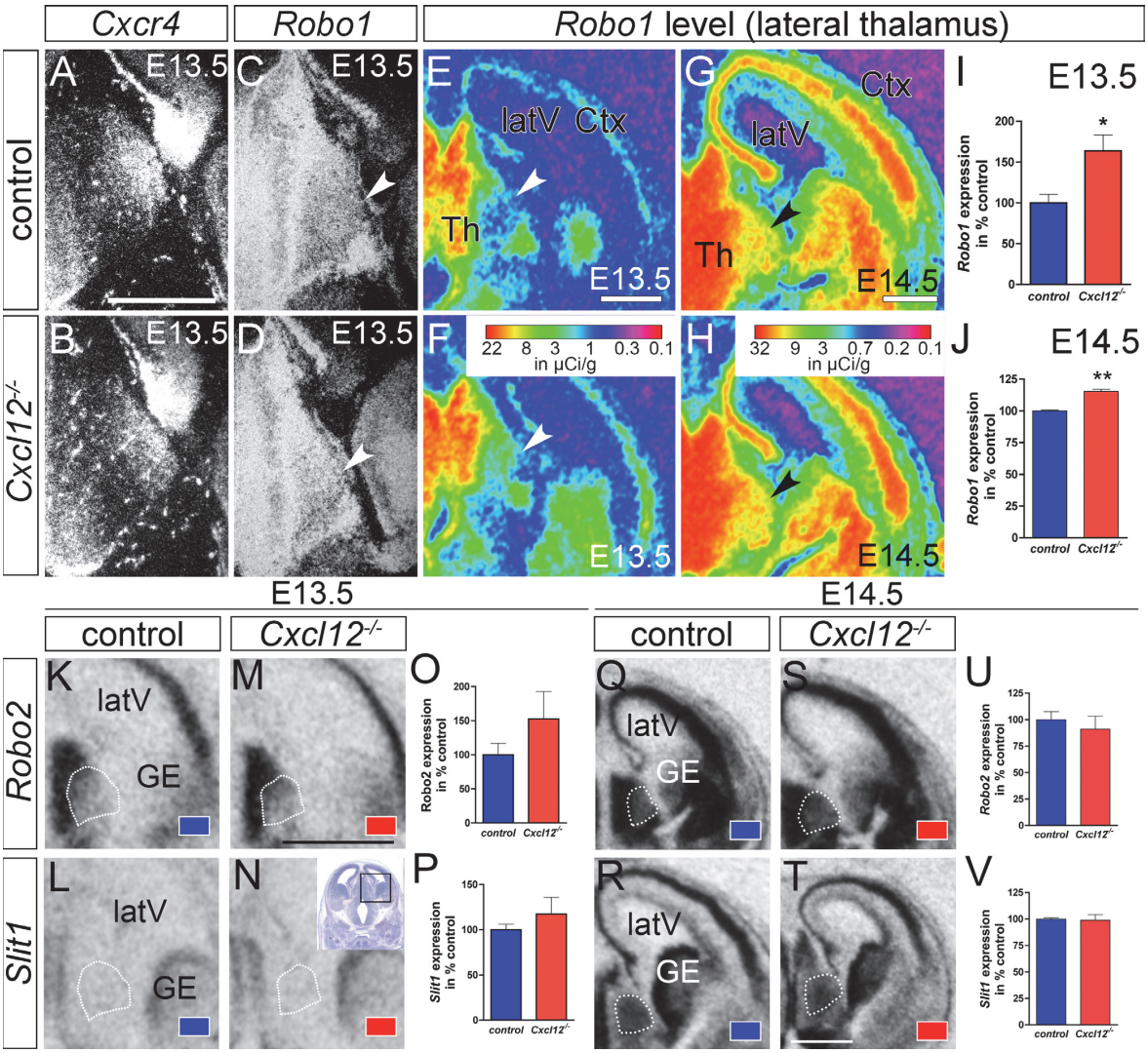

Figure 7. CXCL12 suppresses *Robo1* expression in the lateral thalamus.

A-D, Darkfield views of emulsion-dipped coronal sections through the thalamus of E13.5 control mice (A,C) and *Cxcl12*$^{-/-}$ littermates (B,D). Sections were *in situ* hybridized with ^{35}S-labeled probes for *Cxcr4* (A,B) and *Robo1* (C,D). *Robo1* signal in the lateral thalamus (arrowheads) is increased in the *Cxcl12*$^{-/-}$ mouse as compared to the control.

E-H, *Robo1* levels are shown as false color displays of calibrated X-ray autoradiograms of hybridized E13.5 (E,F) and E14.5 (G,H) sections from control mice (E,G) and *Cxcl12*$^{-/-}$ littermates (F,H). Arrowheads point to the lateral thalamus. Insets in *F* and *H* show signal intensities in *E,F* and *G,H* as ^{14}C equivalents.

I,J, *Robo1* levels in the lateral thalamus of E13.5 (I) and E14.5 (J) control mice and *Cxcl12*$^{-/-}$ littermates. Data are mean+SEM (E13.5: n=3; E14.5: n=4). *p<0.05, **p<0.01 (*t* tests).

K-N, Q-T, X-ray autoradiograms of coronal sections through the thalamus of control and *Cxcl12*$^{-/-}$ mice at E13.5 (K-N) and E14.5 (Q-T). Specimens were *in situ* hybridized with ^{35}S-labeled probes for, *Robo2* (upper panel) and *Slit1* (lower panel). The inset in *N* shows an H&E-stained coronal section, the box corresponds to the area shown in the autoradiograms in the left panel.

O,P,U,V, Expression levels of *Robo2* (O,U) and *Slit1* (P,V) in the lateral thalamus (see circumscribed area in the autoradiograms) at E13.5 (left panel, n=3) and E14.5 (right panel, n=4). Results were obtained by densitometry on autoradiograms after calibration with a ^{14}C standard. *p<0.05, **p<0.01 (t test). Data are mean+SEM.

Abbreviations: Ctx, cortex; GE, ganglionic eminence; latV, lateral ventricle; Th, thalamus**. Scale bars:** 500 µm in *A,E,*G and 1 mm in *M,T*.

We thus hypothesized that the CXCL12/CXCR4 and Slit/Robo systems might interact to regulate TCA elongation. Given that CXCR4 is highly activated in the lateral thalamus (Figure 3A-E), we first examined if CXCL12/CXCR4 signaling regulates expression of Slit/Robo family members in this region. Matched coronal sections from *Cxcl12*$^{-/-}$ mutants and control littermates were hybridized with ^{35}S-labeled probes for *Cxcr4*, *Robo1*, *Robo2* and *Slit1*. Darkfield microscopy revealed stronger *Robo1* signal in the *Cxcr4*-expressing lateral thalamus of E13.5 *Cxcl12*$^{-/-}$ mice as compared to controls (arrowheads in Figure 7C,D). Remarkably, the thalamic area exhibiting changed *Robo1* expression corresponded to the *Cxcr4*-expressing area (Figure 7A,B). CXCL12 deficiency did not obviously affect *Robo2* and *Slit1* expression in the E13.5 thalamus. To confirm these observations, we conducted an additional experiment at E14.5 and measured intensities of the hybridization signals in E13.5 and E14.5 lateral thalami on x-ray autoradiograms after density calibration with a ^{14}C standard exposed together with the hybridized specimens (Figure 7E-V; false color reproductions of density-calibrated autoradiograms of *Robo1* are shown in Figure 7E-H). This confirmed that mRNA levels of *Slit1* and *Robo2* were not changed (Figure 7K-V) while the *Robo1* level was significantly increased by 63.8% in E13.5 *Cxcl12*$^{-/-}$ ($p=0.014$) and by 15.3% in E14.5 *Cxcl12*$^{-/-}$ embryos ($p=0.004$) as compared to littermate controls (Figure 7I,J, *t* tests). These data indicate that CXCL12/CXCR4 signaling suppresses mRNA expression of the Slit1 receptor Robo1 in the lateral thalamus.

Next, we tested for functional interaction between CXCL12 and Slit1/Robo pathways by examining axon outgrowth in E13.5 *Cxcr4*-GFP^{+} thalamic explant cultures (Figure 8A). Stimulation with CXCL12 dose-dependently increased outgrowth of *Cxcr4*-GFP^{+} axons ($p<0.0001$, ANOVA, Figure 8B). Outgrowth was significantly increased by 17.0% and by 30.5% after 20 nM and 40 nM CXCL12, respectively ($p<0.001$ vs. non-stimulated control, Bonferroni´s post-hoc test). Slit1 was applied at 5 nM (Figure 8C), a concentration that evokes growth cone collapse in dissociated thalamic cultures (Mire et al., 2012). This concentration had no significant effect when applied alone (vehicle: 100±1.0, Slit1: 102.2±1.2%) but almost abolished the growth promoting effect of CXCL12 (CXCL12: 125.7±1.4%, CXCL12+Slit1: 108.3±1.5%, $p<0.001$, ANOVA and post-hoc test), indicating that Slit1/Robo signaling inhibits outgrowth of *Cxcr4*-GFP^{+} axons from thalamic explants downstream of CXCL12/CXCR4.

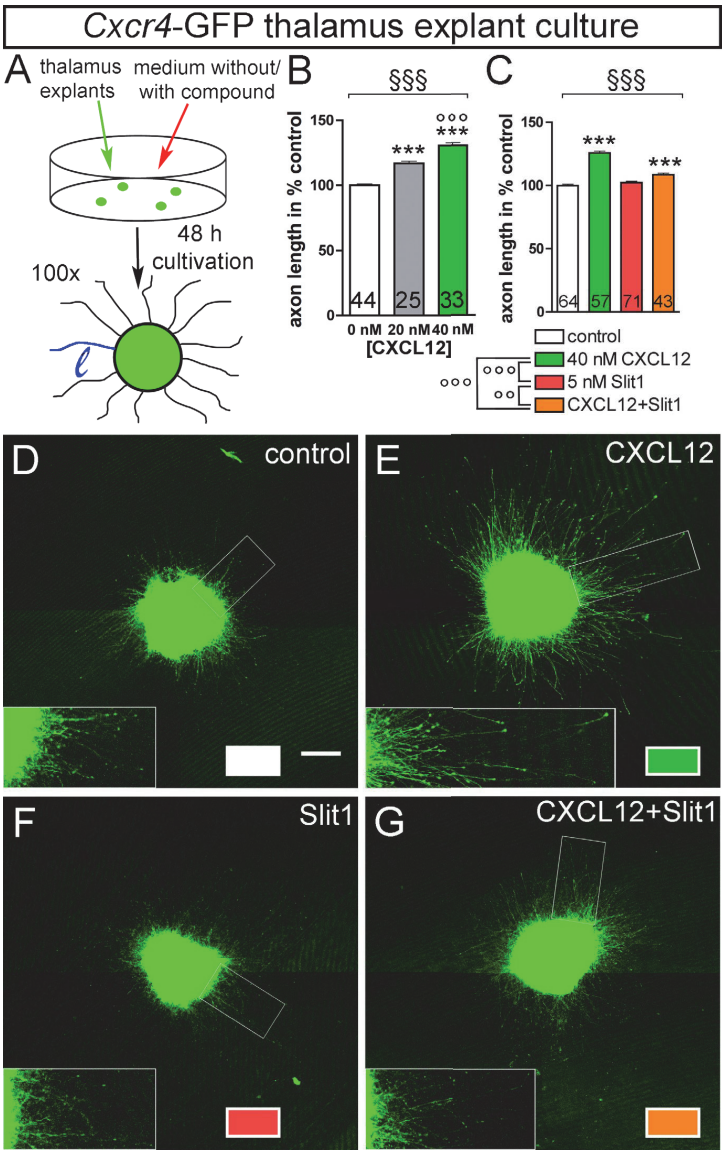

Figure 8. Slit1 functionally antagonizes CXCL12-promoted axonal growth in thalamus explants.

A, Scheme illustrating experimental procedure of explant culturing. Thalamus explants were prepared from E13.5 *Cxcr4*-GFP reporter mice and cultured for two days in medium supplemented with vehicle, CXCL12 or Slit1. In each explant, length (l) of the 20 longest axons was measured.

B,C CXCL12 dose-dependently stimulates axonal growth. Slit1 (5 nM) reduces axonal growth induced by 40 nM CXCL12. Axon length is expressed as mean+SEM (number of analyzed explants is given in bar).

D-G, Representative *Cxcr4*-GFP^{+} thalamus explants stimulated with culture medium (control, D), 40 nM CXCL12 (E), 5 nM Slit1 (F) and CXCL12/Slit1 (G). Inserts show boxed area under 2x magnification. Color bars in D-G represent color code used in B,C.

Statistics: $^{§§§}p<0.0001$ (ANOVA). $^{°°°}$,***$p<0.001$; $^{°°}$,**$p<0.01$ (Bonferroni's post-hoc test compared to *control and °between treatments as indicated). **Scale bar:** 200 µm in D.

DISCUSSION

While considerable progress has been made in identifying molecules that guide migrating cortical interneurons and the forming thalamocortical tract (Hernandez-Miranda et al., 2010; Kolodkin and Tessier-Lavigne, 2011; Grant et al., 2012; Price et al., 2012), little is known how sufficient supply with interneurons and TCAs to newly generated cortical neurons is coordinated. A large proportion of cortical PNs arises from IPCs residing in the SVZ (Kolodkin and Tessier-Lavigne, 2011; Vasistha et al., 2014). Intriguingly, the SVZ is traversed by migrating interneurons and ingrowing TCAs during the peak of cortical neurogenesis (Hevner et al., 2004; Mire et al., 2012). We show that deletion of *Cxcl12* in IPCs reduces progression of ingrowing TCAs and disrupts migration of interneurons in the SVZ/IZ (Figure 9). Mechanistically, we uncover mutual interaction between CXCL12/CXCR4 and Slit/Robo pathways, which is likely to regulate TCA growth *in vivo*. To our knowledge, this is

the first report to provide evidence that CXCL12 regulates axonal growth within the developing mammalian brain. Our findings imply that, while amplifying cortical neurons (Martinez-Cerdeno et al., 2006), IPCs enable efficient progression of TCAs and interneurons by releasing CXCL12.

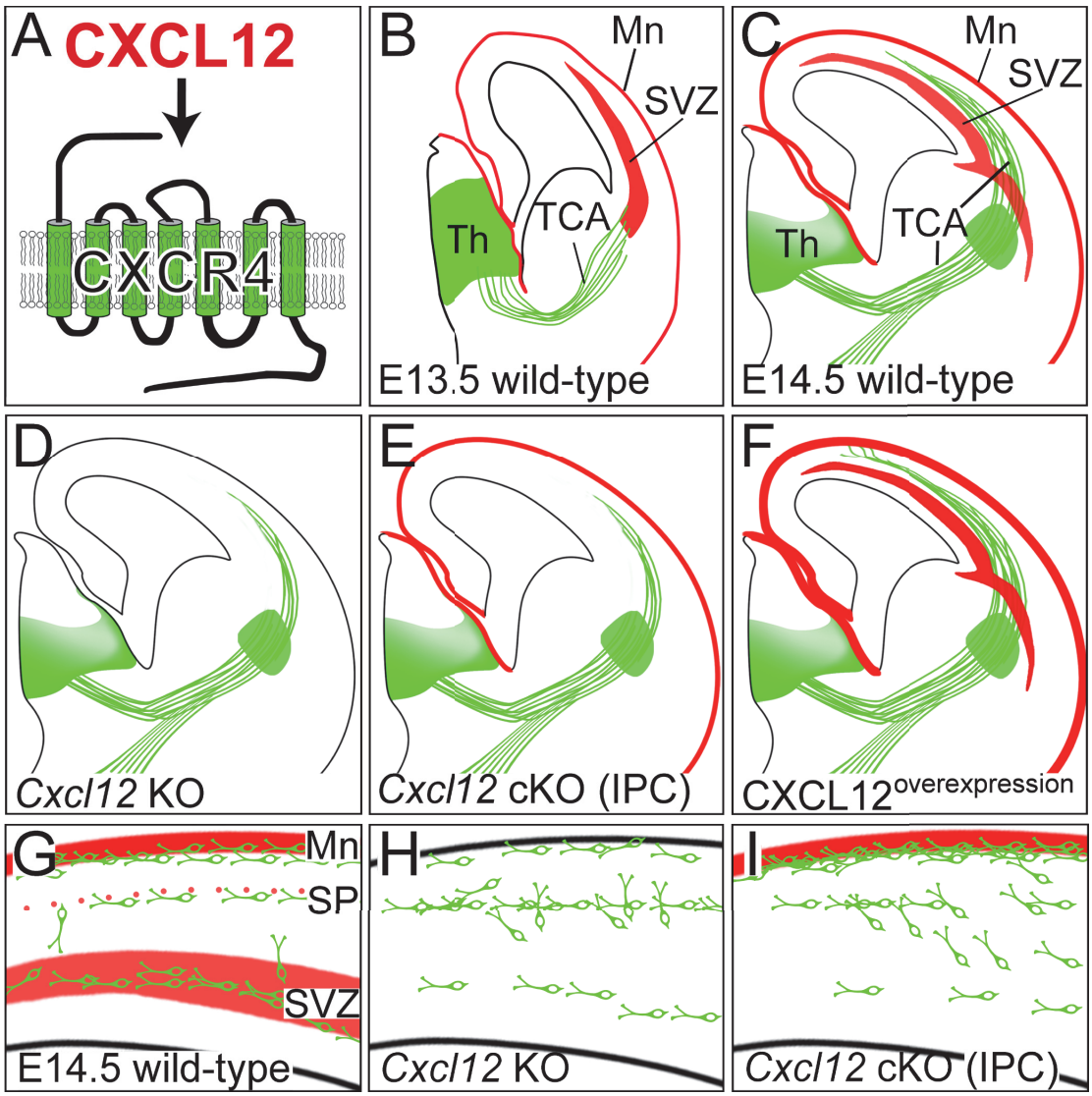

Figure 9. Summarizing scheme.
A, *Cxcl12*-expressing structures are shown in red and *Cxcr4*-expressing structures in green. **B,C,** *Cxcr4*-GFP$^+$ TCAs reach the cortical SVZ at E13.5 and traverse most of the cortex between E13.5 and E14.5.
D,E, Intracortical growth of *Cxcr4*-GFP$^+$ TCAs is reduced in *Cxcl12*-deficient mice (*Cxcl12* KO, *D*) and in conditional mutants lacking CXCL12 in IPCs (*Cxcl12* cKO, *E*).
F, Orthotopic CXCL12 overexpression promotes intracortical growth of *Cxcr4*-GFP$^+$ TCAs and leads to premature cortical plate (CP) invasion and abnormal targeting of the MZ.
G, CXCR4-positive interneurons migrate in the MZ, subplate (SP) and SVZ.
H, In *Cxcl12*-deficient mice, interneurons accumulate in the CP/SP area.
I, After *Cxcl12* ablation in IPCs, interneurons leave the SVZ and seek upper cortical layers.
Abbreviations: Mn, meninges; MZ, marginal zone; SP, subplate; SVZ, subventricular zone; TCA, thalamocortical afferents; Th, thalamus.

CXCL12 promotes axonal growth in the mammalian brain

While CXCL12 effects on axonal growth and axonal guidance have been studied in *in vitro* models (Arakawa et al., 2003; Chalasani et al., 2003; Pujol et al., 2005; Heskamp et al., 2013), CXCL12 effects on axonal development within the mammalian brain were not previously resolved. We thus examined expression and function of the CXCL12/CXCR4/CXCR7 signaling module in the embryonic thalamus and the forming thalamocortical projection, one of the major intracerebral axonal tracts. CXCR4 receptors are present in cell bodies and growing axons of E12 – E14 thalamocortical projection neurons. The second CXCL12 receptor, CXCR7, is present to the progenitor domain but absent from the differentiation field in the E12 – E14 thalamus. Examination of the CXCR4 activation state showed that CXCL12 - most likely emanating from the meninges covering the thalamus - signals through CXCR4 to the cell bodies of thalamic neurons. Regions in the thalamus, diencephalon, prethalamus and internal capsule that are traversed by TCAs are largely *Cxcl12*-negative, suggesting that CXCL12 is not involved in TCA guidance in these regions. Consistently, misrouting of TCAs is not observed in embryos lacking CXCL12. Instead, we present several lines of evidence that CXCL12 stimulates TCA growth. First, CXCL12 enhances axonal growth in thalamus explants. Second, orthotopic CXCL12 overexpression in CXCL12-RFP-transgenic mice leads to increased intracortical TCA growth. Third, intracortical TCA growth is severely attenuated in *Cxcl12*$^{-/-}$ mice. As the thalamic *Gbx2* and *Sox2* expression domains, which give rise to thalamocortical relay neurons (Chatterjee and Li, 2012), appear normal in *Cxcl12*$^{-/-}$ embryos, it seems unlikely that reduced TCA growth is due to defective thalamus formation. A previous study addressing the role of CXCL12 in axonal development in a mammalian system demonstrated that mesenchymal CXCL12 guides CXCR4^{+} motor axons once they grow out of the neural tube (Lieberam et al., 2005). Our finding that CXCL12 promotes intracranial growth of long-ranged axons connecting the thalamus to the late-developing cortex thus unveils a new aspect of CXCL12 function.

Meningeal-derived CXCL12 regulates *Robo1* expression in lateral thalamic nuclei

Slit/Robo signaling is critically involved in TCA guidance and growth (Bagri et al., 2002; Andrews et al., 2006; Lopez-Bendito et al., 2007). Recently, neuronal activity was shown to increase growth speed of TCAs by suppressing *Robo1* transcription in early thalamocortical neurons (Mire et al., 2012). According to this report, spontaneous activity and TCA growth

decrease at E14. This corresponds to the timepoint when *Cxcr4* expression decreases in the tMZ, suggesting a link between CXCL12/CXCR4 signaling, neuronal activity and *Robo1* transcription in the thalamus. In support of this, we found that CXCR4 receptors are highly activated and that *Cxcl12* deficiency increases *Robo1* transcript levels in the lateral thalamus. As the thalamus is *Cxcl12*-negative before E15, *Cxcl12*-expressing meninges are the most likely CXCL12 source to activate CXCR4 and regulate *Robo1* in the lateral thalamus. Consistently, CXCR4 activation in the lateral thalamus was abrogated in $Cxcl12^{-/-}$ mice but not in $Cxcl12cKO^{Tbr2\text{-}Cre}$ mice that maintain meningeal *Cxcl12* expression.

Slit1 functionally antagonizes CXCL12 signaling downstream of CXCR4

We observed that adding Slit1 to thalamus explants reduces CXCL12-promoted but not normal axonal growth, suggesting that Slit1/Robo signaling inhibits an axon growth-promoting pathway downstream of CXCR4. Among the four Robo receptors, Robo1 and Robo2 are *bona fide* Slit receptors expressed in the developing thalamus (Bagri et al., 2002), which implies these two receptors in the Slit1 effect *in vitro*. In the embryonic brain, *Robo1* deficiency (Andrews et al., 2006) and CXCL12-overexpression result in a similar TCA phenotype characterized by accelerated growth and premature contact to cortical targets. As conditional deletion of *Cxcl12* in IPCs reduces TCAs progression in the cortex, it is reasonable to assume that IPC-derived CXCL12 promotes TCA growth by stimulating CXCR4 at the growth cone. Slit1 is strongly expressed in the cortical plate and the predominant Slit in the embryonic cortex (Bagri et al., 2002). It is thus conceivable that Slit1/Robo1 growth-inhibiting and CXCL12/CXCR4 growth-promoting pathways converge in growth cones of TCAs progressing in the cortex. Focal adhesion kinase (FAK) is highly enriched in axonal growth cones and integrates growth-promoting, growth-inhibiting and repulsive signals including those from the Slit/Robo system (Navarro and Rico, 2014). Although little is known about FAK modulation by CXCR4 in neuronal cells, FAK activation by CXCR4 has been shown in hematopoietic cells (Wang et al., 2000). We thus propose that IPC-derived CXCL12 and PN-derived Slit1 converge on FAK in TCA growth cones to oppositely regulate intracortical growth.

IPC-derived CXCL12 supports tangential migration in the SVZ

The marginal zone and SVZ/IZ are major routes of interneurons migrating tangentially in the cortex (Wonders and Anderson, 2006). *Cxcl12* mRNA is expressed in the meninges, in the SVZ and, to lesser extent, in the subplate (Stumm et al., 2003; Stumm et al., 2007; Abe et al., 2014). The chemokine is attractant for interneurons and supports tangential migration in the cortex. Both CXCL12 receptors, CXCR4 and CXCR7, are involved in this process (Sanchez-Alcaniz et al., 2011; Wang et al., 2011; Abe et al., 2014). The prominent *Cxcl12* mRNA signal in the SVZ is localized in IPC (Tiveron et al., 2006). This is a unique constellation because *Cxcl12* expression in neuronal progenitors has not been reported in other regions of the embryonic brain. It has been proposed that CXCL12 from IPCs provides a molecular link between IPCs and interneurons (Tiveron et al., 2006; Sessa et al., 2010). This hypothesis was based on the observation that *Pax6* and *Tbr2* mutants exhibit reduced *Cxcl12* expression in the SVZ and abnormal interneuron migration. However, there are severe defects in cortical development in *Tbr2* and *Pax6* mutants and absence of these crucial factors affects expression of a large set of genes other than *Cxcl12*. It was therefore not clear if perturbed interneuron migration in *Tbr2* and *Pax6* mutants is indeed caused by defective *Cxcl12* expression in IPCs (Tiveron et al., 2006; Arnold et al., 2008; Sessa et al., 2010; Georgala et al., 2011; Elsen et al., 2013). By showing that interneurons deviate from their normal migration in the SVZ to accumulate in the CP and MZ in mice with a selective deletion of *Cxcl12* in IPCs, we now provide proof that IPC-derived CXCL12 is essential for interneuron migration in the SVZ.

Conclusion

Evolution of the cortex is characterized by dramatic expansion of cortical size in mammals. This is enabled by IPC-mediated amplification of cortical neurons. Our study demonstrates that IPC-derived CXCL12 promotes TCA growth and interneuron migration, providing evidence that paracrine signaling mechanisms exist that coordinate neuronal amplification and invasion of the cortex by extrinsic cell and axon elements.

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AUTHOR CONTRIBUTION

R.S. and P.A. designed the study. P.A. carried out experiments, analyzed data and drafted the manuscript. P.A. and Z.M. conducted the DiI tracing study. R.S. participated in data analysis and wrote the final manuscript. S.A., Y.S.T. and D.M.L. provided mice and helped to write the manuscript.

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4.2 Manuskript II – CXCR7 prevents excessive CXCL12-mediated down-regulation of CXCR4 in migrating cortical interneurons

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Ralf Stumm, Philipp Abe und Wiebke Müller haben das Projekt entwickelt. Philipp Abe, Wiebke Müller und Dagmar Schütz haben die Experimente durchgeführt, die Daten ausgewertet und das Manuskript zusammen mit Ralf Stumm entworfen. Philipp Abe hat alle histologischen (IHC, ISH) und *ex vivo* (Lebendzellbeobachtung, Schnittkultur) Studien durchgeführt. Wiebke Müller und Dagmar Schütz haben den biochemischen Teil und die HEK293 Experimente ausgeführt. Ralf Stumm war in die Datenanalyse involviert und hat das finale Manuskript geschrieben. Fabienne MacKay, Marcus Thelen und Penglie Zhang haben Reagenzien bereitgestellt und halfen, die Experimente zu etablieren.

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RESEARCH REPORT

CXCR7 prevents excessive CXCL12-mediated downregulation of CXCR4 in migrating cortical interneurons

Philipp Abe[1,*], Wiebke Mueller[1,*], Dagmar Schütz[1], Fabienne MacKay[2], Marcus Thelen[3], Penglie Zhang[4] and Ralf Stumm[1,‡]

ABSTRACT

The CXCL12/CXCR4 signaling pathway is involved in the development of numerous neuronal and non-neuronal structures. Recent work established that the atypical second CXCL12 receptor, CXCR7, is essential for the proper migration of interneuron precursors in the developing cerebral cortex. Two CXCR7-mediated functions were proposed in this process: direct modulation of β-arrestin-mediated signaling cascades and CXCL12 scavenging to regulate local chemokine availability and ensure responsiveness of the CXCL12/CXCR4 pathway in interneurons. Neither of these functions has been proven in the embryonic brain. Here, we demonstrate that migrating interneurons efficiently sequester CXCL12 through CXCR7. CXCR7 ablation causes excessive phosphorylation and downregulation of CXCR4 throughout the cortex in mice expressing CXCL12, but not in CXCL12-deficient animals. *Cxcl12*$^{-/-}$ mice lack activated CXCR4 in embryonic brain lysates and display a similar interneuron positioning defect as *Cxcr4*$^{-/-}$, *Cxcr7*$^{-/-}$ and *Cxcl12*$^{-/-}$;*Cxcr7*$^{-/-}$ animals. Thus, CXCL12 is the only CXCR4-activating ligand in the embryonic brain and deletion of one of the CXCL12 receptors is sufficient to generate a migration phenotype that corresponds to the CXCL12-deficient pathway. Our findings imply that interfering with the CXCL12-scavenging activity of CXCR7 causes loss of CXCR4 function as a consequence of excessive CXCL12-mediated CXCR4 activation and degradation.

KEY WORDS: CXCL12, CXCR4, CXCR7 (ACKR3), Interneuron migration, Atypical chemokine receptor, Cortical development, Mouse, Cajal-Retzius cell

INTRODUCTION

The chemokine CXCL12 regulates cell migration and homing processes in the immune system, hematopoietic system, brain, and other tissues (Stumm and Hollt, 2007; Miller et al., 2008; Tiveron and Cremer, 2008; Lewellis and Knaut, 2012). It mediates its effects through canonical G protein-coupled CXCR4 and through CXCR7 (Bleul et al., 1996; Oberlin et al., 1996; Balabanian et al., 2005; Burns et al., 2006). The latter is also referred to as atypical chemokine receptor 3 (ACKR3) because it does not signal through G proteins and elicits no chemotaxis (Thelen and Thelen, 2008). Instead, CXCR7 sequesters CXCL12 from the extracellular environment and, thus, functions as a CXCL12 scavenger (Venkiteswaran et al., 2013; Boldajipour et al., 2008; Luker et al., 2010; Naumann et al., 2010; Hoffmann et al., 2012). Like other heptahelical receptors, including CXCR4, CXCR7 is also capable of modulating downstream pathways through β-arrestin (Cheng et al., 2000; Kalatskaya et al., 2009; Rajagopal et al., 2010). Thus, CXCL12 signaling is subject to particularly complicated regulation. Unraveling the underlying mechanisms will facilitate an understanding of CXCL12-mediated cell guidance and the development of new treatment strategies for cell migration disorders.

Defective CXCR4 regulation has recently been implicated in the perturbed intracortical migration of GABAergic interneurons in genetic schizophrenia models (Meechan et al., 2012; Toritsuka et al., 2013). Cortical interneurons originate in the ganglionic eminences in the ventral telencephalon (Anderson et al., 1997; Marín and Rubenstein, 2001; Hernández-Miranda et al., 2010). They seek the cortical marginal zone (MZ) and subventricular/intermediate zone (SVZ/IZ), where their migration depends on CXCL12 emanating from meningeal cells and pyramidal cell progenitors (Tiveron et al., 2006; Tanaka et al., 2009; Sessa et al., 2010; Zarbalis et al., 2012). Most interneurons express both CXCL12 receptors, and interneurons deficient for CXCR4 or CXCR7 display opposite motility defects (Wang et al., 2011). This suggests that distinct CXCR4-dependent and CXCR7-dependent pathways might exist in interneurons: CXCR4 attracts cells towards CXCL12 through a G protein-dependent pathway (Lysko et al., 2011), whereas CXCR7 modulates MAP kinases through β-arrestin (Wang et al., 2011).

Having observed that CXCR7-deficient interneurons display severely reduced CXCR4 levels and loss of CXCR4 function (Sánchez-Alcañiz et al., 2011), we proposed that CXCR7 regulates interneuron migration by preserving CXCR4. This is consistent with the observation that a reduction in CXCR4 disrupts CXCL12-mediated interneuron guidance (Meechan et al., 2012). Given that proper CXCR4 regulation is essential in interneurons, we examined how the two receptors cooperate in this system. We first tested whether the similar interneuron positioning defects in *Cxcr4*$^{-/-}$ and *Cxcr7*$^{-/-}$ mutants (Sánchez-Alcañiz et al., 2011; Wang et al., 2011) reflect a fully CXCL12-defective pathway by assessing interneuron layering in *Cxcr4*$^{-/-}$, *Cxcr7*$^{-/-}$ and *Cxcl12*$^{-/-}$ mice. This showed that inactivation of one of the CXCL12 receptors is sufficient to generate the phenotype observed in mice lacking CXCL12 signaling. We then demonstrated that migrating interneurons sequester CXCL12 through CXCR7, which supports the concept that CXCR7 acts as a CXCL12 scavenger in these cells. Finally, we conducted biochemical and histochemical studies, from which we conclude that loss of CXCR7 leads to excessive CXCL12-mediated activation and downregulation of CXCR4 in interneurons.

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RESULTS AND DISCUSSION

Cxcl12$^{-/-}$, *Cxcr4*$^{-/-}$ and *Cxcr7*$^{-/-}$ mice display similar interneuron layering defects

To examine whether aberrant interneuron layering in *Cxcr4*$^{-/-}$ and *Cxcr7*$^{-/-}$ mutants corresponds to a partial or complete CXCL12 signaling defect, we examined patterns of *Lhx6* (a marker for MGE-derived interneurons), *Cxcr4* and *Cxcr7* expression in the cortex of E14.5 *Cxcl12*$^{-/-}$, *Cxcr4*$^{-/-}$ and *Cxcr7*$^{-/-}$ mouse embryos by *in situ* hybridization (Fig. 1). Cell counting showed that the numbers of *Lhx6*$^{+}$ and Cxcr4^{+}/Neo^{+} cells were reduced in the MZ and SVZ and increased in the lower cortical plate/subplate (CP/SP) in the three knockout lines compared with control littermates (Fig. 1Ae-g,Ce-g). *Cxcr7*$^{+}$ cells, as counted in *Cxcl12*$^{-/-}$, *Cxcr4*$^{-/-}$ and control littermates (Fig. 1Be,f), exhibited similar abnormal distributions to *Lhx6*$^{+}$ cells and Cxcr4^{+}/Neo^{+} cells in these knockouts. The matching patterns of *Lhx6*, *Cxcr4* and *Cxcr7* were expected because *Lhx6*$^{+}$ cells constitute the main *Cxcr4*- and *Cxcr7*-expressing population in the embryonic cortex (Sánchez-Alcañiz et al., 2011; Wang et al., 2011). We then compared the laminar distribution of *Lhx6*$^{+}$ cells in *Cxcl12*$^{-/-}$, *Cxcr4*$^{-/-}$ and *Cxcr7*$^{-/-}$ mice using ANOVA. This revealed no significant differences, suggesting that deletion of one of the CXCL12 receptors is

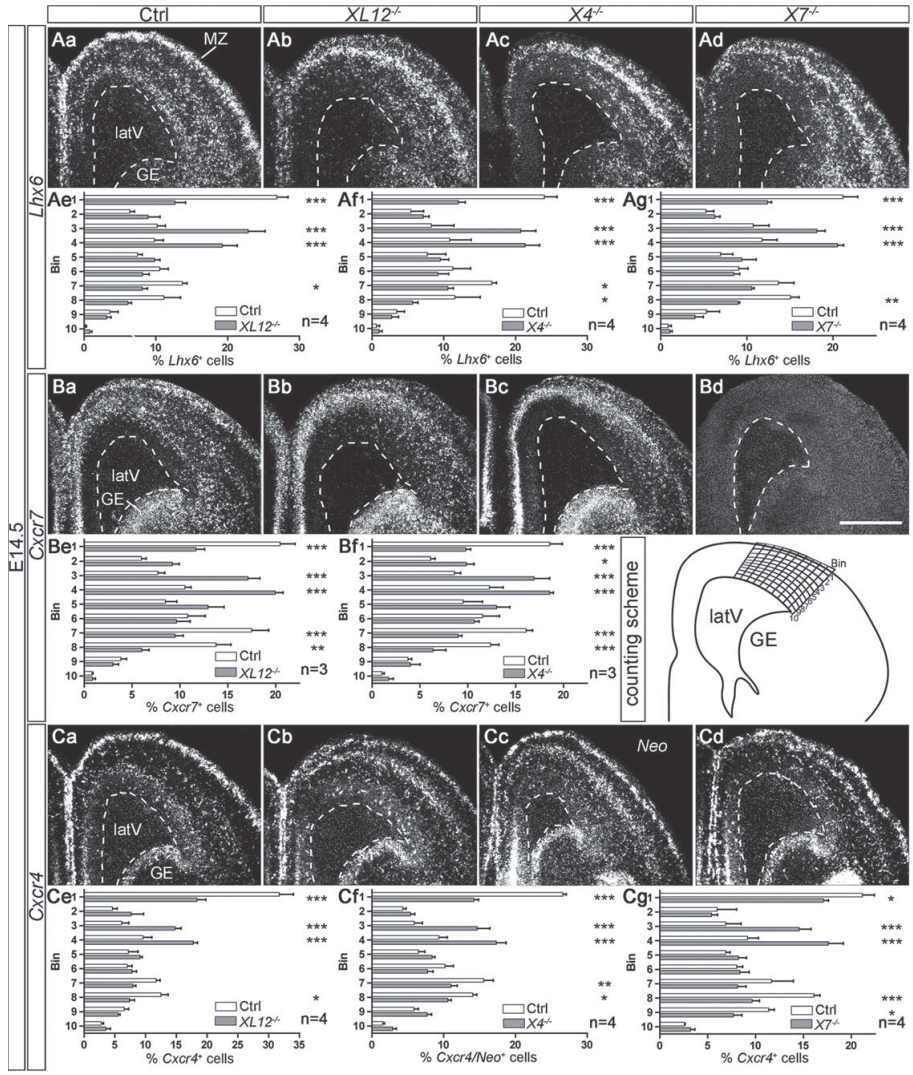

Fig. 1. Similar interneuron distribution defects in E14.5 *Cxcl12*$^{-/-}$, *Cxcr4*$^{-/-}$ and *Cxcr7*$^{-/-}$ mice. Dark-field photographs show the dorsal telencephalon in *in situ* hybridization preparations with ^{35}S-labeled probes for *Lhx6* (Aa-d), *Cxcr7* (Ba-d) and *Cxcr4*/*Neo* (Ca-d) in control (Ctrl), *Cxcl12*$^{-/-}$ (*XL12*$^{-/-}$), *Cxcr4*$^{-/-}$ (*X4*$^{-/-}$) and *Cxcr7*$^{-/-}$ (*X7*$^{-/-}$) mice. The *Neo* probe detects *Cxcr4* gene-derived transcripts in *Cxcr4*$^{-/-}$ mice (Cc). *Cxcr7* transcripts are not detected in *Cxcr7*$^{-/-}$ mutants (Bd). (Ae-g,Be,f,Ce-g) Labeled cells were counted in ten cortical bins (see counting scheme). Proportions per bin (percentage of all counted cells) are presented as mean+s.e.m. Mutants and control littermates were compared using two-way ANOVA and Bonferroni's post-test. *P≤0.05, **P≤0.01 and ***P≤0.001. latV, lateral ventricle; GE, ganglionic eminence; MZ, marginal zone. Scale bar: 500 µm in Bd for Aa-d, Ba-d, Ca-d.

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sufficient to generate the interneuron layering defect observed in animals lacking CXCL12.

In the cortex, reelin (*Reln*)$^{+}$ Cajal-Retzius cells represent a second major *Cxcr4*-expressing population that depends on CXCL12/CXCR4 signaling (Stumm et al., 2003; Borrell and Marín, 2006; Paredes et al., 2006). Cell counting in the parietal cortex revealed a reduction of these cells in the MZ (supplementary material Fig. S1A), but only a few ectopic cells in deep cortical layers in *Cxcl12*$^{-/-}$, *Cxcr4*$^{-/-}$ and *Cxcr7*$^{-/-}$ mice. This finding is consistent with the reported function of CXCL12 and CXCR4 in Cajal-Retzius cell dispersion in the MZ (Borrell and Marín, 2006). Given that cortical *Reln*$^{+}$ cells rarely express *Cxcr7* (Schönemeier et al., 2008), our data point to the possibility that CXCR7 exerts some non-autonomous influence on this process.

CXCR7 sequesters CXCL12 in migrating interneurons

By examining red fluorescent protein (RFP) immunoreactivity in the cortex of E14.5 mice expressing CXCL12-RFP fusion protein under the *Cxcl12* promoter (Jung et al., 2009), we identified strongly stained punctae in all cortical layers. The highest densities were observed in the MZ, SP and SVZ/IZ (Fig. 2Aa,B; supplementary material Fig. S1D). This pattern differs markedly from that of *Cxcl12* expression, which is restricted to the meninges and SVZ at E14.5 (Tiveron et al., 2006). Since *Rfp* transcripts in the cortex of CXCL12-RFP embryos faithfully recapitulate the *Cxcl12* mRNA pattern in wild types (supplementary material Fig. S1B), we reasoned that the observed CXCL12-RFP^{+} punctae reflect endocytosed and not nascent CXCL12-RFP. We therefore quantified the number of RFP^{+} punctae (of size >0.5 μm^2) in the cortex of CXCL12-RFP mice lacking the CXCL12 scavenger CXCR7 (Fig. 2Ab). Automated counting using ImageJ revealed a 74.2±9.0% reduction in *Cxcr7*$^{-/-}$;CXCL12-RFP mice as compared with CXCL12-RFP controls (n=4; P<0.01, Student's t-test). Such signal reduction was not observed after *Cxcr4* deletion (Fig. 2Ac). This indicates that most of the strong punctate RFP signal in CXCL12-RFP mice was due to CXCR7-mediated accumulation of CXCL12-RFP. The meninges, in which *Cxcl12* is highly expressed, exhibited similar RFP labeling in receptor-deficient and control mice (supplementary material Fig. S1Ca-c), suggesting that RFP signal in this tissue reflects locally expressed CXCL12-RFP. The specificity of RFP immunostaining was controlled in CXCL12-RFP non-transgenic *Cxcr7*-GFP embryos (supplementary material Fig. S1E).

Closer examination of the RFP pattern in *Cxcr4*$^{-/-}$;CXCL12-RFP sections revealed a signal shift towards the lower CP/SP area (supplementary material Fig. S1D). This aberrant pattern is reminiscent of the interneuron layering defect in *Cxcr4*$^{-/-}$ embryos, suggesting that RFP signal was contained within interneurons. As cortical interneurons do not express *Cxcl12* (Tiveron et al., 2006; Wang et al., 2011), we hypothesized that they sequester CXCL12-RFP. To test this, we generated double-transgenic mice (*Cxcr7*-GFP; CXCL12-RFP) and performed simultaneous live cell imaging of GFP and RFP in E14.5 cortical slices (Fig. 2B; supplementary material Movie 1). Analysis of the confocal images revealed that 96% of *Cxcr7*-GFP^{+} interneurons contained RFP^{+} punctae. To assess whether CXCR7 mediates CXCL12-RFP accumulation in interneurons, we immunostained for RFP in E14.5 *Cxcr7*$^{-/-}$;CXCL12-RFP;*Cxcr7*-GFP and CXCR7-expressing CXCL12-RFP;*Cxcr7*-GFP embryos (Fig. 2Ca-d), and quantified GFP/RFP overlap in the cortex (Mander's coefficient) using ImageJ. In the latter group, 60.3±7.1% of total RFP was present in *Cxcr7*-GFP^{+} interneurons (Fig. 2Ca,b). In the absence of CXCR7, the RFP signal that was contained in *Cxcr7*-GFP^{+}

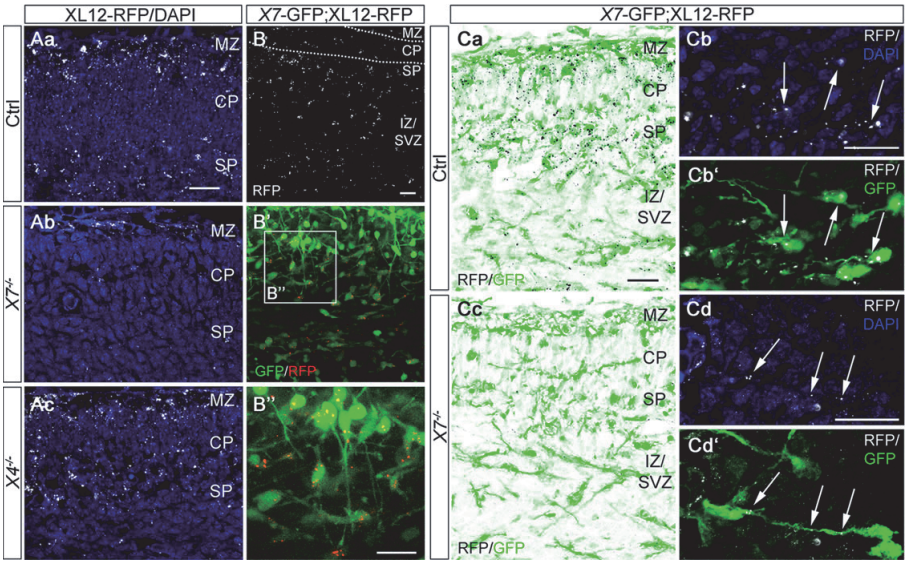

Fig. 2. CXCR7 accumulates CXCL12-RFP in interneurons. Confocal images show RFP in the cortex of E14.5 CXCL12-RFP transgenic mice (XL12-RFP; false colors for RFP are indicated). (A) Immunostained RFP and DAPI in control (Aa), XL12-RFP;*Cxcr7*$^{-/-}$ (*X7*$^{-/-}$, Ab) and XL12-RFP;*Cxcr4*$^{-/-}$ (*X4*$^{-/-}$, Ac) mice. (B-B″) Native RFP (B) and overlay of native GFP/RFP (B′) in a slice from a *Cxcr7*-GFP;XL12-RFP embryo (*X7*-GFP;XL12-RFP). (B″) Magnification of the boxed area in B′ demonstrates RFP^{+} punctae in live GFP^{+} cells. These cells show migration modes characteristic of interneurons (supplementary material Movie 1). (Ca-d) Immunostained RFP in *X7*-GFP;XL12-RFP (Ca,b) and *X7*-GFP;XL12-RFP;*Cxcr7*$^{-/-}$ (Cc,d) mice. Higher magnification images show RFP/DAPI (Cb,d) and RFP/GFP overlays (Cb′,d′). Arrows indicate internalized CXCL12-RFP in *Cxcr7*-GFP+ interneurons. MZ, marginal zone; CP, cortical plate; SP, subplate; IZ/SVZ, intermediate/subventricular zone. Scale bars: 20 μm.

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interneurons was decreased by 79.9±7.2% (P<0.0001, Student's t-test; n=4; Fig. 2Cc,d).

We then examined whether *Cxcr7*-GFP$^+$ interneurons are capable of internalizing exogenous fluorophore-coupled CXCL12 (CXCL12-565). After validating receptor/CXCL12-565 interaction in transfected HEK293 cells (supplementary material Fig. S2A), we incubated telencephalic slices from E14.5 *Cxcr7*-GFP mice for 30 min with CXCL12-565. CXCL12, the CXCR4 antagonist AMD3100 and the CXCR7 antagonist CCX771 (Zabel et al., 2009) were co-incubated in adjacent slices. Confocal imaging of native fluorescence revealed bright intracellular clusters of CXCL12-565 in *Cxcr7*-GFP$^+$ interneurons that received CXCL12-565 alone or CXCL12-565/AMD3100 (supplementary material Fig. S2Ba,c). Slices that received CXCL12-565 and an excess of CXCL12 showed no CXCL12-565 signal, and slices that received CXCL12-565/CCX771 showed faint CXCL12-565 signal, in *Cxcr7*-GFP$^+$ interneurons (supplementary material Fig. S2Bb,d). Collectively, our analyses of CXCL12-RFP localization and CXCL12-565 uptake demonstrate that interneurons efficiently internalize CXCL12 via CXCR7.

CXCR7 gauges CXCL12-induced CXCR4 activation in telencephalic cultures

Next, we examined the influence of CXCR7 on CXCL12-induced activation and downregulation of CXCR4 in telencephalic neurons using immunoblots of E14.5 cultures. Blots were reacted with the anti-CXCR4 antibody UMB-2 that recognizes the non-phosphorylated C-terminal epitope 343-352, which undergoes S346/347-phosphorylation upon CXCL12 stimulation (Mueller et al., 2013). Thus, UMB-2 detects inactive CXCR4 in non-dephosphorylated and total CXCR4 in dephosphorylated samples. Consistently, lysate from cultures that received CXCL12 for 30 min showed a strong signal difference between non-dephosphorylated

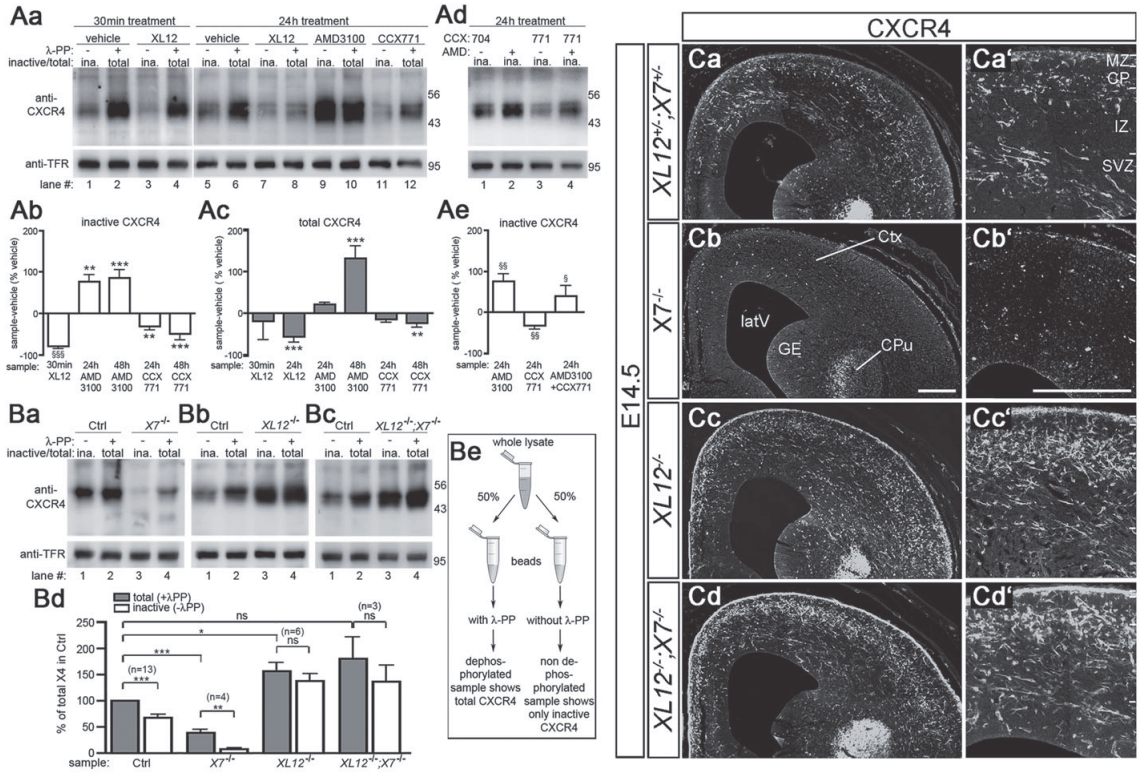

Fig. 3. CXCR7 regulates CXCL12-promoted activation and downregulation of CXCR4. (A,B) Immunoblots analyzing the expression level and activation state of CXCR4 in lysates from E14.5 telencephalic neurons (Aa-e) and E15.5 *Cxcr7*$^{-/-}$ (*X7*$^{-/-}$), *Cxcl12*$^{-/-}$ (*XL12*$^{-/-}$) and *Cxcl12*$^{-/-}$;*Cxcr7*$^{-/-}$ (*XL12*$^{-/-}$;*X7*$^{-/-}$) brains (Ba-d). Blots were reacted with the anti-CXCR4 antibody UMB-2, which detects the non-phosphorylated C-terminus, and with anti-transferrin receptor (TFR) to demonstrate equal loading. (Be) Lysates were split into two aliquots and purified with wheatgerm lectin agarose beads. Aliquots dephosphorylated using Lambda protein phosphatase (λ-PP) show total CXCR4, whereas untreated aliquots show non-phosphorylated/inactive CXCR4 (ina.). (A) CXCL12 (XL12), AMD3100 (AMD), CCX771, and inactive compound (CCX704) were added for the indicated period. (Ab,c,e) For quantification of compound-induced changes in CXCR4, the difference between the UMB-2/TFR signal ratios of compound-treated cultures (sample) and vehicle-treated sister cultures (vehicle) was expressed as a percentage of UMB-2/TFR of the vehicle. Compound-treated and corresponding vehicle-treated groups were compared using one-way ANOVA and Bonferroni's post-test (*) or Student's t-test (§) (three to ten independent repeats). (Ba-c) Immunoblots compare pooled lysates from two knockouts with pooled lysates from two wild-type or heterozygous littermates (Ctrl). (Bd) Each CXCR4/TFR ratio was expressed as a percentage of the CXCR4/TFR ratio of the corresponding λ-PP-treated control before repeats were averaged and analyzed using Student's t-test. Data are mean+s.e.m. $^{*,\S}P\leq0.05$, $^{**,\S\S}P\leq0.01$ and $^{***}P\leq0.001$; ns, not significant. (C) UMB-2 immunofluorescence in coronal head sections of a single E14.5 litter (genotypes as specified in the figure). Higher magnifications (Ca′-d′) show the cerebral cortex. CPu, caudate putamen; GE, ganglionic eminence; latV, lateral ventricle; Ctx, cortex. Scale bars: 200 µm.

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and dephosphorylated aliquots, which is characteristic of CXCR4 activation (Fig. 3Aa, lanes 3 and 4). Continuous CXCL12 treatment reduced the CXCR4 level (Fig. 3Aa, lanes 6 and 8; Fig. 3Ac, 24 h XL12), indicating CXCL12-induced CXCR4 downregulation.

Cultures that were maintained over a 24-48 h period without medium exchange exhibited considerable CXCR4 activation (Fig. 3Aa, lanes 5 and 6). Activation was caused by endogenous CXCL12, as 24 h treatment with CXCR4 antagonist (AMD3100) rendered virtually all CXCR4 receptors inactive (Fig. 3Aa, lanes 9 and 10). Continuous antagonist treatment caused an increase in total CXCR4 as compared with vehicle-treated control cultures (Fig. 3Ac, 48 h AMD3100), indicating prevention of CXCL12-induced CXCR4 downregulation. When CXCR7-mediated uptake of endogenous CXCL12 was blocked by long-term CCX771 treatment, CXCR4 levels decreased (Fig. 3Aa, lanes 5, 11 and 6, 12; Fig. 3Ab,c, 24 h and 48 h CCX771). The CCX771 effect was blocked by AMD3100 (Fig. 3Ad, lanes 1, 3, 4; Fig. 3Ae), which suggests that it was CXCL12 mediated. Collectively, these experiments provide evidence that CXCR7 antagonism augments CXCL12-induced activation and downregulation of CXCR4 in telencephalic cultures.

CXCR7 prevents excessive CXCR4 activation by CXCL12

We then assessed whether CXCR7 influences CXCL12-mediated activation and downregulation of CXCR4 in the embryonic brain. We analyzed *Cxcr7*$^{-/-}$, *Cxcl12*$^{-/-}$ and *Cxcl12*$^{-/-}$;*Cxcr7*$^{-/-}$ mice by immunoblotting and immunofluorescence methods. Immunoblots were reacted with UMB-2 (Fig. 3B) and cortical sections with UMB-2 or the phosphorylation-insensitive anti-CXCR4 antibody 2B11 (Fig. 3C; supplementary material Fig. S3A). In all immunoblots, the controls showed a marked difference between total and inactive CXCR4 (Fig. 3Ba-c, lanes 1 and 2; Fig. 3Bd, total versus inactive in control group), indicating substantial CXCR4 activation by endogenous ligand. In *Cxcl12*$^{-/-}$ mice, almost all CXCR4 receptors were in the inactive state (Fig. 3Bb, lanes 3 and 4), suggesting that CXCL12 is the only CXCR4-activating ligand in the embryonic brain. Furthermore, CXCR4 signal was stronger in brain lysates (Fig. 3Bb, lanes 2 and 4; Fig. 3Bd, control versus *Cxcl12*$^{-/-}$) and cortical sections (Fig. 3Ca,c) from these mutants. Given that the level of *Cxcr4* mRNA was apparently unaltered in *Cxcl12* knockouts (Fig. 1Ca,b), these findings provide evidence that a substantial proportion of CXCR4 in the embryonic brain becomes downregulated after being activated by CXCL12.

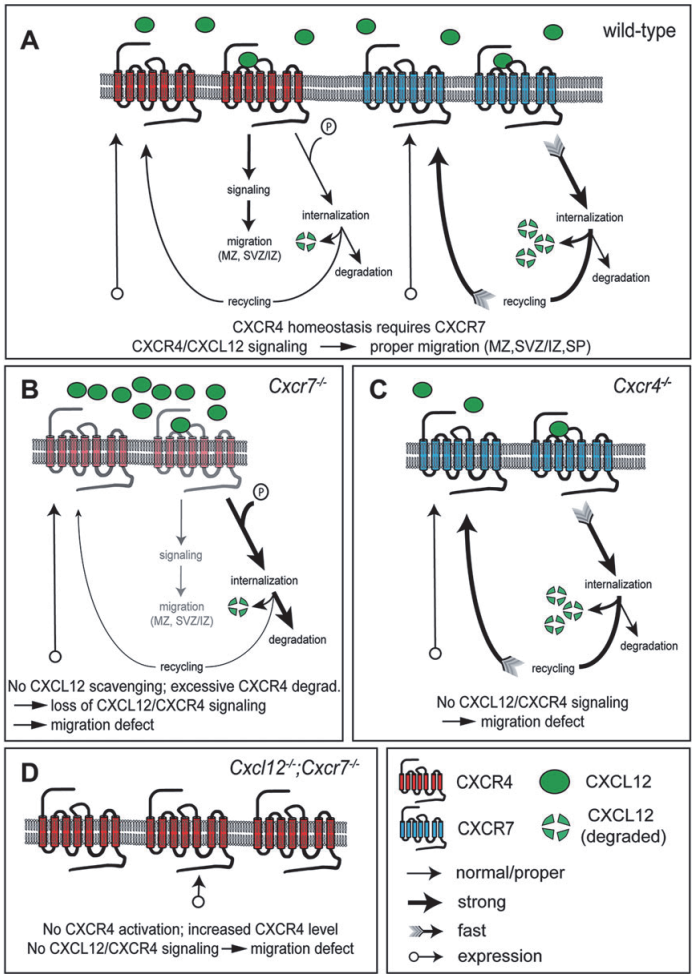

Fig. 4. Scheme illustrating functions of CXCR4 and CXCR7 in migrating cortical interneurons. (A) CXCL12 induces signaling and phosphorylation of CXCR4 as well as internalization of the CXCL12/CXCR4 complex. Internalized CXCR4 is recycled or degraded. CXCL12/CXCR4 signaling supports interneuron migration in the MZ and SVZ/IZ. Synthesis and degradation of CXCR4 are at equilibrium. CXCR7 mediates rapid internalization and degradation of CXCL12. (B) Absence of CXCR7 leads to extracellular CXCL12 accumulation and excessive CXCR4 activation and degradation. Loss of CXCR4 results in insufficient CXCL12/CXCR4 signaling and defective interneuron migration. (C) Lack of CXCL12/CXCR4 signaling causes defective interneuron migration. (D) The CXCR4 level is increased in the absence of CXCL12. CXCR7 deficiency does not cause excessive CXCR4 degradation because CXCR4 does not become phosphorylated when CXCL12 is not present. Lack of CXCL12/CXCR4 signaling causes defective interneuron migration.

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As reported previously (Sánchez-Alcañiz et al., 2011), CXCR4 was hardly detectable in brain lysates and cortical sections from *Cxcr7*$^{-/-}$ mice (Fig. 3Ba, lanes 2 and 4; Fig. 3Bd, control group versus *Cxcr7*$^{-/-}$; Fig. 3Ca,b). We have now generated *Cxcl12*$^{-/-}$;*Cxcr7*$^{-/-}$ mice to test whether the loss of CXCR4 in *Cxcr7* mutants is mediated by CXCL12, and found that the activation state and expression level of CXCR4 in *Cxcl12*$^{-/-}$;*Cxcr7*$^{-/-}$ mice resembled those of *Cxcl12*$^{-/-}$ animals: almost all CXCR4 receptors were inactive (Fig. 3Bc, lanes 3 and 4) and the level of CXCR4 was increased compared with controls (Fig. 3Bc, lanes 2 and 4; Fig. 3Bd, control group versus *Cxcl12*$^{-/-}$;*Cxcr7*$^{-/-}$). CXCR4 immunostaining in the cortex was indistinguishable between *Cxcl12*$^{-/-}$;*Cxcr7*$^{-/-}$ and *Cxcl12*$^{-/-}$ mice: both cohorts showed a similar increase in CXCR4 signal as compared with the control (Fig. 3Ca,c,d) and a similar layering defect of CXCR4-immunoreactive neurons (supplementary material Fig. S3B).

Our findings in *Cxcr7*$^{-/-}$ and *Cxcl12*$^{-/-}$;*Cxcr7*$^{-/-}$ animals demonstrate that the embryonic brain contains sufficient CXCL12 to induce near complete CXCR4 degradation unless CXCR7-dependent scavenger activity prevents excessive CXCR4 activation by CXCL12. These findings and conclusions are summarized in Fig. 4.

MATERIALS AND METHODS

Animals and histochemistry

Animal procedures were in accordance with German and EU guidelines. Established mouse lines, *in situ* hybridization and immunohistochemical procedures, probes and antibodies were used (Memi et al., 2013; Stumm et al., 2002, 2003; Sánchez-Alcañiz et al., 2011). Transcripts from the *Neomycin* resistance cassette of *Cxcr4* mutants (Zou et al., 1998) were detected with a *Neo* probe cloned by PCR using *Cxcr4*$^{+/-}$ genomic DNA and 5′-ATGGGATCGGCCATTGAAC-3′ and 5′-TCAGAAG-AACTCGTCAAG-3′ primers.

CXCL12-565 uptake

Recombinant Atto565-tagged CXCL12 (CXCL12-565) (Yang et al., 1999) was labeled with a C-terminal ybbR13 tag (George et al., 2004; Zhou et al., 2007). Human embryonic kidney (HEK293) cells were transiently transfected as described (Hoffmann et al., 2012). Surface CXCR4 and CXCR7 receptors were immunostained in fixed non-permeabilized cells with N-terminal antibodies 2B11 (BD Biosciences) and 11G8 (Zabel et al., 2009), respectively. HEK293 cells and cortical slices were incubated with 20 nM CXCL12-565 at 37°C, washed, and fixed before confocal imaging.

Immunoblotting

Neuronal cultures (25×10^6 cells/dish) were prepared from E14.5 telencephalic vesicles and grown in Neurobasal medium/B27 supplement (Invitrogen). Compounds (20 nM CXCL12, Peprotech; 6 µM AMD3100, Sigma; 1 µM CCX771/CCX704, ChemoCentryx) were added 4 h after seeding in 6 ml fresh medium. Immunoblotting, detection and analysis were as described (Sánchez-Alcañiz et al., 2011).

Live cell imaging

Coronal brain slices (300 µm) were cut on a Vibratome (Leica), placed in 35 mm glass-bottom dishes (MatTek) and embedded in collagen (3 mg/ml, BD Biosciences). After adding DMEM lacking Phenol Red (Gibco), slices were transferred to the 37°C/5% CO_2 tissue incubation chamber attached to an LSM510 Meta inverted confocal microscope (Zeiss). The following live cell acquisition setup was used: EC Plan-Neofluor 20×/0.50 M27 objective, time series (interval: 10 min), *z*-stack acquisition, maximum intensity projection processing using Zen software (Zeiss). Statistical tests are specified in the figure legends (one, two and three symbols indicate $P\leq0.05$, $P\leq0.01$, and $P\leq0.001$, respectively).

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Competing interests

The authors declare no competing financial interests.

Author contributions

R.S., P.A. and W.M. designed the study. P.A., W.M. and D.S. carried out experiments, analyzed data and helped draft the manuscript. R.S. participated in data analysis and wrote the final manuscript. M.T., P.Z. and F.M. provided reagents and helped to establish the experiments.

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Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.104224/-/DC1

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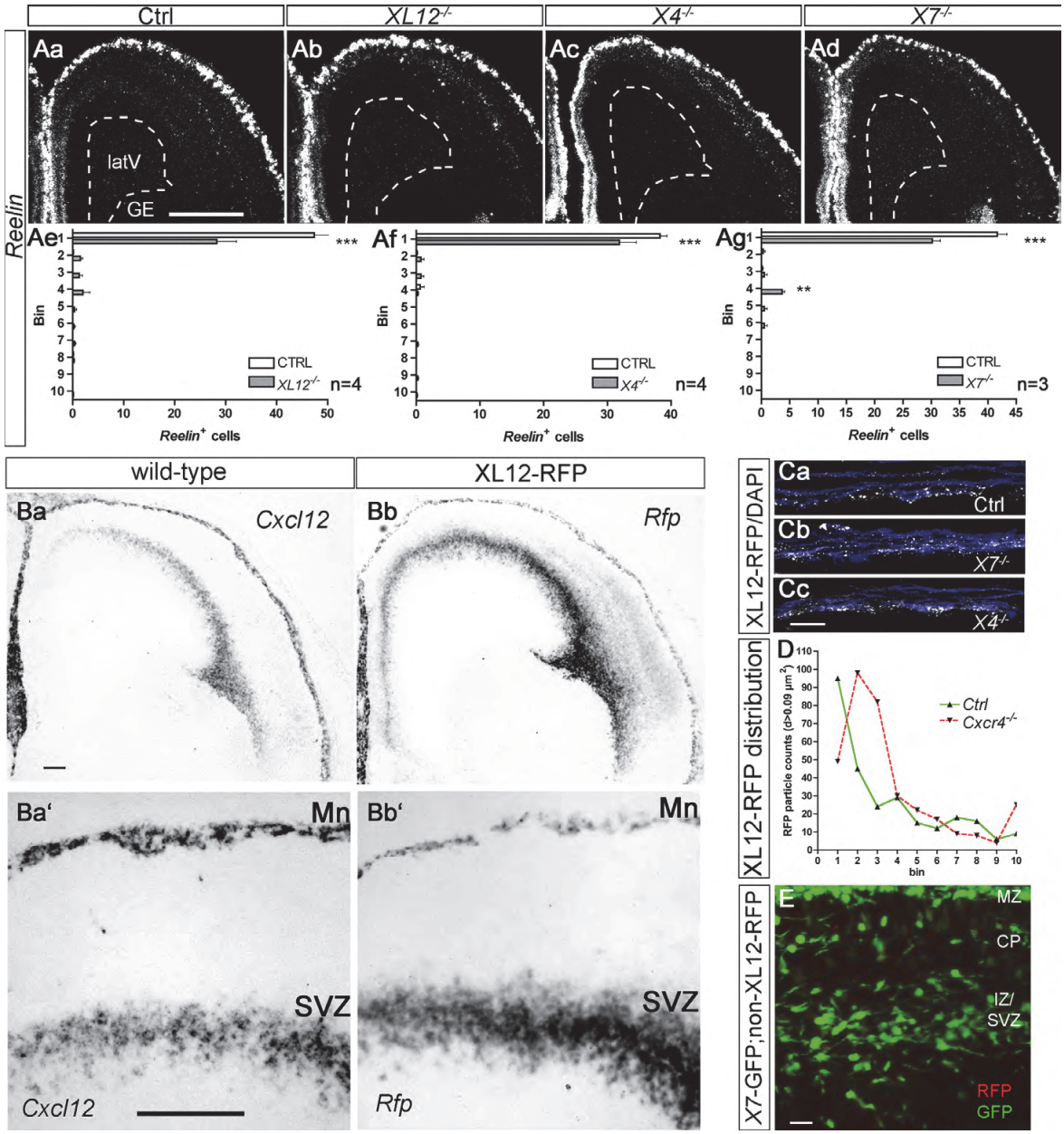
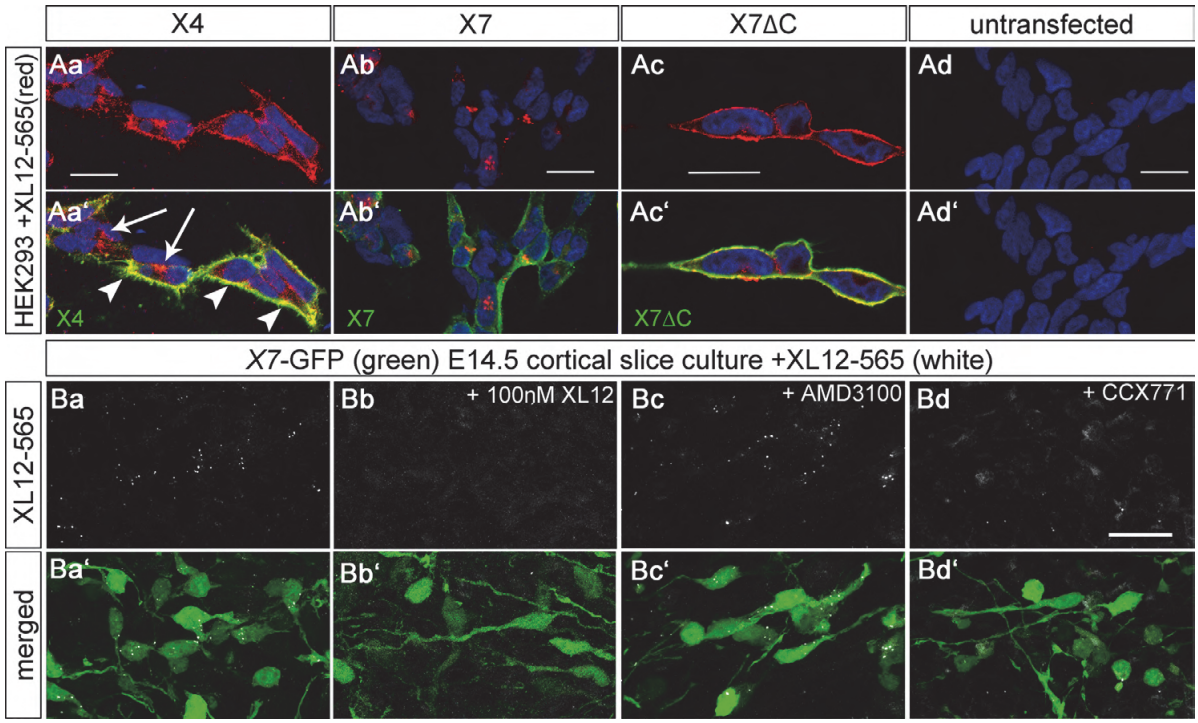
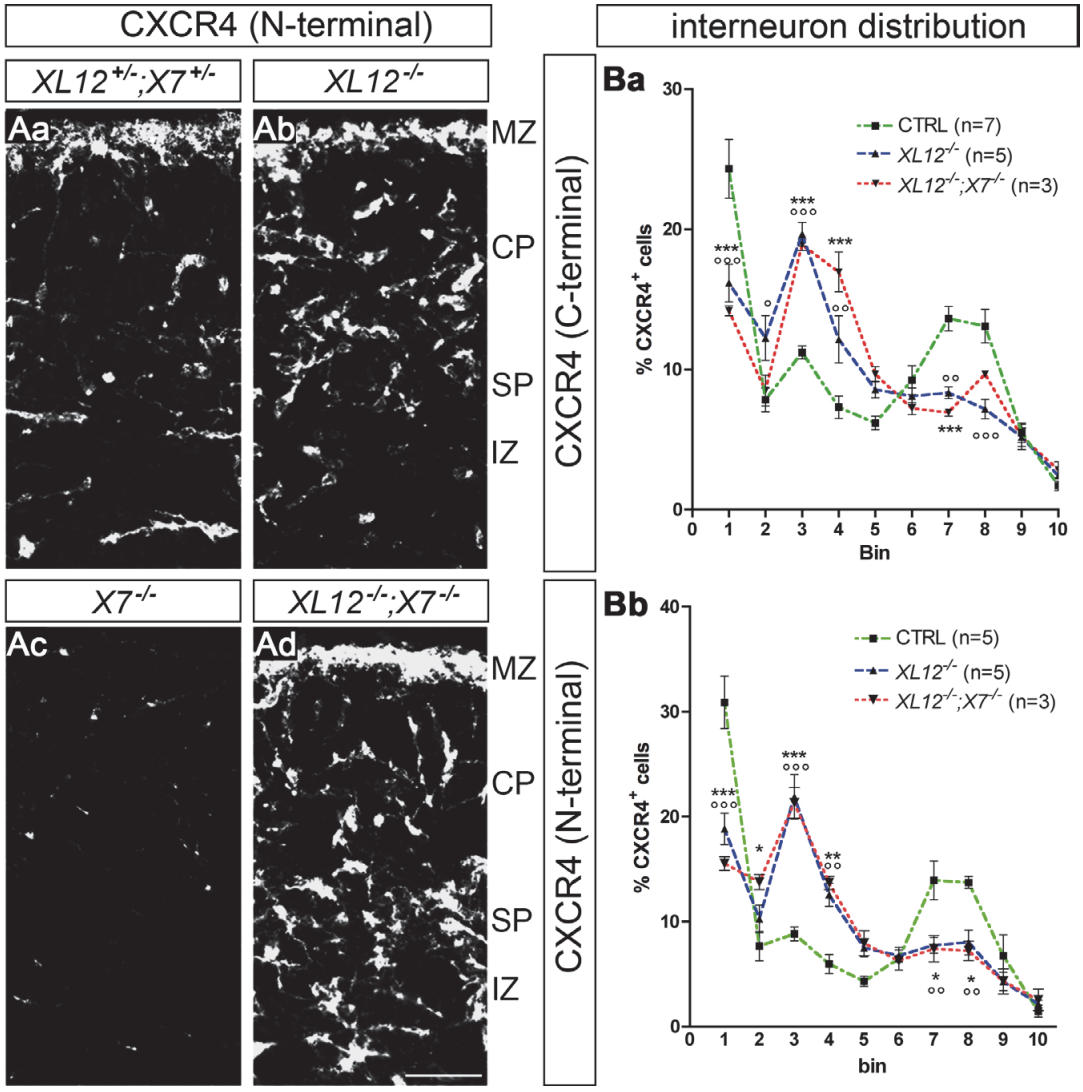


Supplemental Figure 1. AaA d, Dark-field photographs of the dorsal telencephalon in emulsion-dipped coronal head sections after *in situ* hybridizations with a ^{35}S-labeled probe for *Reln* transcripts in control (Ctrl, Aa), *Cxcl12*$^{-/-}$ (*XL12*$^{-/-}$, Ab), *Cxcr4*$^{-/-}$ (*X4*$^{-/-}$, Ac), and *Cxcr7*$^{-/-}$ (*X7*$^{-/-}$, Ad) mice. **AeA g**, Graphs show numbers of *Reln*$^{+}$ cells in 10 cortical bins (lateral cortex, see Figure 1 for counting scheme). Mutants were compared to control littermates using two-way ANOVA and Bonferroni´s post-hoc test (*Cxcl12*$^{-/-}$ and *Cxcr4*$^{-/-}$, n=4; *Cxcr7*$^{-/-}$, n=3). **Ba,Bb**, Bright-field photographs of the dorsal telencephalon in coronal head sections after *in situ* hybridizations with digoxigenin-labeled probes for *Cxcl12* and *Rfp* in a wild-type (Ba) and a CXCL12-RFP (Bb) mouse, respectively. **Ba',Bb'**, Details of the cortex. *Cxcl12* and *Cxcl12-Rfp* transcripts exhibit similar patterns characterized by strong expression in the meninges and the subventricular/intermediate zone. **CaC c**, Confocal images show immunostained RFP (white) in the meninges of E14.5 CXCL12-RFP (Ca, control), CXCL12-RFP;*Cxcr7*$^{-/-}$ (Cb, *X7*$^{-/-}$), and CXCL12-RFP;*Cxcr4*$^{-/-}$ (Cc, *X4*$^{-/-}$) mice. **D**, CXCL12-RFP signal distribution in E14.5 CXCL12-RFP (Ctrl) and CXCL12-RFP;*Cxcr4*$^{-/-}$ cortices. In the *Cxcr4* knockout the RFP signal shifts towards the CP/SP area (bins #2,3). **E,** Dual immunofluorescence for RFP/GFP in the cortex of an E14.5 *Cxcr7*-GFP mouse lacking the CXCL12-RFP transgene (non-XL12-RFP). RFP signal is absent in the overlay of the confocal RFP/GFP images. GE, ganglionic eminence; latV, lateral ventricle; Mn, meninges. Scale bars: Aa, 500 µm; Ba, Ba´, 200 µm; Cc,E, 20 µm.



Supplemental Figure 2. A, Validation of CXCL12-565/receptor interaction. Transiently transfected HEK293 cells were incubated for 30 min at 37°C with CXCL12-565. Surface receptors were visualized in non-permeabilized cells with N-terminal antibodies (green). DAPI is shown in blue. **Aa**, In CXCR4-expressing cells (X4), most of the recovered CXCL12-565 (red) is colocalized with CXCR4 at the cell surface (arrowheads) and only a small fraction of the compound is internalized (arrow). **Ab**, In CXCR7-expressing cells (X7), virtually all of the recovered CXCL12-565 is clustered inside the cells. **Ac**, Transfectants of CXCR7ΔC (X7ΔC) that lacks the C-terminus and fails to internalize (Zabel et al., 2009) show CXCL12-565 exclusively at the cell surface. **Ad**, In untransfected cells, CXCL12-565 is not recovered. **AaA d**, The images represent single confocal planes. It is concluded that CXCR7 rapidly internalizes CXCL12-565 whereas CXCR4 readily binds the compound but mediates less effective CXCL12-565 uptake than CXCR7. The qualitative results with CXCL12-565 correspond to quantitative results obtained with radiolabeled CXCL12 in a similar setting (Hoffmann et al., 2012). **B,** CXCL12-565 uptake in *Cxcr7*-GFP$^+$ interneurons. Cortical slices from E14.5 *Cxcr7*-GFP (*X7*-GFP) transgenic embryos were incubated for 30 min at 37°C with CXCL12-565 and additional compounds as indicated. Native fluorescence, imaged by confocal microscopy, is shown for CXCL12-565 in white (Ba-Bd) and for CXCL12-565 plus GFP in the white/green overlay (Ba´-Bd´). Intracellular accumulation of CXCL12-565 in interneurons (Ba,Ba´) is blocked by excess non-fluorescent CXCL12 (XL12, Bb,Bb´) and the CXCR7 ligand CCX771 (Bd,Bd´), but not by the CXCR4 antagonist AMD3100 (Bc,Bc´).



Supplemental Figure 3. Similar layering defect of CXCR4⁺ cells in the cortex of E14.5 *Cxcl12*$^{-/-}$ and *Cxcl12*$^{-/-}$;*Cxcr7*$^{-/-}$ mice. A, CXCR4 was detected with phospho-insensitive N-terminal 2B11 antibody in coronal sections of an E14.5 litter (genotypes of littermates are specified in the Figure). **Ba,Bb,** UMB-2-immunoreactive cells (Ba) and 2B11-immunoreactive (Bb) were counted in 10 cortical bins in *Cxcl12*$^{-/-}$, *Cxcl12*$^{-/-}$;*Cxcr7*$^{-/-}$, and control mice (heterozygous or wild-type). Proportions per bin (% of all counted cells) are presented as mean±s.e.m. Mutants were compared to control mice using two-way ANOVA and Bonferroni´s post-test (^{O}*Cxcl12*$^{-/-}$; **Cxcl12*$^{-/-}$;*Cxcr7*$^{-/-}$). Note that abnormal layering of CXCR4⁺ cells is similar in the two mutants. Scale bar in Ad: 50 µm.

Movie 1. Virtually all *Cxcr7*$^{+}$ interneurons (96%) accumulate CXCL12R FP while migrating through the cortex. Live cell imaging of *Cxcr7*-GFP;CXCL12-RFP double transgenic E14.5 embryo. Confocal image series of the lateral cortex (magnification: 200x; time: 15x10 min interval, z-stack: processed by maximum intensity projection with ZEN 2008 software). CP, cortical plate; IZ, intermediate zone; MZ, marginal zone; SVZ, subventricular zone.

4.3 Manuskript III – CXC chemokine receptor 7 (CXCR7) affects the migration of GnRH neurons by regulating CXCL12 availability

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Ralf Stumm, John G Parnavelas, Fani Memi und Anna Cariboni haben das Projekt entwickelt. Fani Memi und Philipp Abe haben die Experimente durchgeführt und die Daten zusammen mit Ralf Stumm und John G Parnavelas ausgewertet. John G Parnavelas, Ralf Stumm und Fani Memi haben das finale Manuskript geschrieben. Fabienne MacKay und Ralf Stumm haben Reagenzien und analytische Werkzeuge zur Verfügung gestellt.

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Development/Plasticity/Repair

CXC Chemokine Receptor 7 (CXCR7) Affects the Migration of GnRH Neurons by Regulating CXCL12 Availability

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Gonadotropin-releasing hormone (GnRH) neurons are neuroendocrine cells, located in the hypothalamus, that play an essential role in mammalian reproduction. These neurons originate in the nasal placode and migrate during embryonic development, in association with olfactory/vomeronasal nerves, first in the nose, then through the cribriform plate to enter the forebrain, before settling in the hypothalamus. One of the molecules required for their early migration in the nose is the chemokine CXCL12, which is expressed in the embryonic nasal mesenchyme in an increasing ventral to dorsal gradient, presumably guiding GnRH neurons toward the forebrain. Mice lacking CXCR4, the receptor for CXCL12, exhibit defective GnRH cell movement and a significant reduction in their number, suggesting that CXCL12/CXCR4 signaling is important in the migration and survival of these neurons. Here, we investigated the role of the more recently identified second CXCL12 receptor, CXCR7, in GnRH neuron development. We demonstrate that CXCR7 is expressed along the migratory path of GnRH neurons in the nasal cavity and, although not expressed by GnRH neurons, it affects their migration as indicated by the ectopic accumulation of these cells in the nasal compartment in *CXCR7*$^{-/-}$ mice. Absence of CXCR7 caused abnormal accumulation of CXCL12-RFP at CXCR4-positive sites in the nasal area of CXCL12-RFP-transgenic mice and excessive CXCL12-dependent intracellular clustering of CXCR4 in GnRH neurons, suggesting internalization. These findings imply that CXCR7 regulates CXCL12 availability by acting as a scavenger along the migratory path of GnRH neurons and, thus, influences the migration of these cells in a noncell-autonomous manner.

Introduction

Gonadotropin-releasing hormone (GnRH) neurons are a small group of hypothalamic cells that control mammalian reproduction by secreting GnRH decapeptide into the portal vessels of the pituitary. There, GnRH stimulates the production of gonadotropins, which control gonadal function (Merchenthaler et al., 1984). During development, GnRH neurons originate in the nasal placode and migrate in association with olfactory/vomeronasal nerve axons to the hypothalamus (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989). In humans, failure of these neurons to reach the hypothalamus results in infertility (Hardelin, 2001). The mechanisms that guide the migration of GnRH neurons are complex, as they encounter different molecular environments during their migratory journey. Thus, their migration is affected by a wide range of factors (Cariboni et al., 2007b; Wray, 2010; Wierman et al., 2011; Giacobini and Prevot, 2013) including the chemokine CXCL12 (Schwarting et al., 2006; Toba et al., 2008; Casoni et al., 2012), which has an established role in directed cell migration (Tiveron and Cremer, 2008; Lewellis and Knaut, 2012). CXCL12 has been observed in the nasal mesenchyme (NM), whereas its receptor, CXCR4, has been localized in migrating GnRH neurons and olfactory/vomeronasal nerve axons. Further, *CXCR4*-deficient mice exhibit a loss of GnRH neurons and impaired migration, suggesting the importance of CXCL12/CXCR4 signaling in the development of this system (Schwarting et al., 2006; Toba et al., 2008).

Recent work established CXCR7 as the second CXCL12 receptor (Balabanian et al., 2005; Burns et al., 2006). Unlike CXCR4, however, CXCR7 fails to signal through $G\alpha_i$ proteins and does not facilitate cell migration in response to CXCL12, suggesting that it is an atypical chemokine receptor (Burns et al., 2006; Boldajipour et al., 2008; Rajagopal et al., 2010). CXCR7 has a 10-fold higher binding affinity to CXCL12 than CXCR4, and its property to rapidly internalize CXCL12 and to degrade large amounts of extracellular chemokine has led to the concept that it acts as a scavenger to control the levels of the chemokine (Luker et al., 2010; Naumann et al., 2010; Hoffmann et al., 2012). In this context, studies in zebrafish (Boldajipour et al., 2008) and mice (Sanchez-Alcaniz et al., 2011) have demonstrated that CXCR7 indeed plays a role in cell migration by regulating CXCL12 pro-

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tein levels available to CXCR4-expressing cells in the process of migration.

Here we investigated the contribution of CXCR7 in CXCL12 signaling in GnRH neuron migration. We found that *CXCR7* is expressed within the NM, especially in its ventral aspect, but not by GnRH neurons or their guiding axons. Mice deficient of *CXCR7* exhibit impaired migration and ectopic GnRH neuron distribution in the nasal area, suggesting that this receptor affects their directed migration in an indirect manner, possibly by regulating CXCL12 availability. Indeed, a CXCL12/RFP fusion protein (CXCL12-RFP) accumulated at different sites in the nasal area of CXCL12-RFP-transgenic mice lacking functional CXCR7 than of CXCL12-RFP-transgenic controls. Furthermore, *CXCR7*-null mice exhibited severely reduced CXCR4 immunoreactivity and excessive intracellular clustering of CXCR4 in the entire nasal area including GnRH neurons, which suggests receptor hyperactivation due to CXCL12 overstimulation. In support of this hypothesis, *CXCL12*$^{-/-}$;*CXCR7*$^{-/-}$ mice displayed lack of CXCR4 internalization and increased CXCR4 levels, pointing to a role for CXCR7 in controlling CXCL12 availability in this system.

Materials and Methods

Animals. To establish mRNA expression patterns for CXCR7, CXCL12, and CXCR4 in the nasal area of wild-type mice, we used pregnant C57BL/6 mice purchased from Charles River. Embryos of transgenic *CXCR7*-eGFP and *CXCR4*-eGFP mice (The Gene Expression Nervous System Atlas project GENSAT; http://www.gensat.org/index.html), and transgenic mice expressing CXCL12-RFP under control of the *CXCL12* promoter (Bhattacharyya et al., 2008), bred on CD1 background, were used for immunohistochemistry. *CXCL12*$^{-/-}$, *CXCR7*$^{-/-}$, and *CXCR4*$^{-/-}$ embryos were obtained by mating heterozygous mice (Nagasawa et al., 1996; Zou et al., 1998; Sierro et al., 2007), which were on C57BL/6 background. *CXCR7*-eGFP;CXCL12-RFP and *CXCR7*$^{-/-}$; CXCL12-RFP mice were obtained by breeding CXCL12-RFP mice with *CXCR7*-eGFP and *CXCR7*$^{+/-}$ animals, respectively. Noon of the day after mating was defined as embryonic day 0.5 (E0.5). All embryos examined in this study were collected regardless of sex. All animal procedures were performed in accordance with UK Animals (Scientific Procedures) Act 1986 and German and EU guidelines and approved by the Thuringer Landesamt fur Lebensmittelsicherheit und Verbraucherschutz (Reg.-Nr. 22-2684-04-02-015/11).

Riboprobes and in situ *hybridization procedure.* The *CXCL12* probe was transcribed from full-length mouse *CXCL12α* cDNA (Tashiro et al., 1993). Probes for mouse *CXCR7* and mouse *CXCR4* corresponded to the receptors coding regions (Stumm et al., 2002; Sanchez-Alcaniz et al., 2011). All described cDNAs were subjected to DNA sequencing for control. Riboprobes were generated from the linearized vector constructs by *in vitro* transcription using digoxigenin-UTP (DIG; Roche) as label. *In situ* hybridization was performed as described previously (Faux et al., 2010). Briefly, embryonic heads were dissected in PBS, pH 7.4, fixed in 4% paraformaldehyde (PFA) made in PBS overnight, followed by cryoprotection in 30% sucrose, treated with diethyl pyrocarbonate (DEPC)/PBS, at 4C for 1 d. Heads were frozen in Tissue-Tek OCT and sectioned coronally at 20 μm. Sections were dried at room temperature (RT) for 2 h, before overnight incubation at 65C in hybridization buffer 1× DEPC-treated sa lts (200 mM NaCl, 5 mM EDTA, 10 mM Tris, pH 7.5, 5 mM $NaH_2PO_4 \cdot 2H_2O$, 5 mM Na_2HPO_4; Sigma-Aldrich), 50% deionized formamide (Ambion), 0.1 mg/ml RNase-free yeast tRNA (Invitrogen), 1× Denhardts (RNase/DNase free; Invitrogen), and 10% dextran sulfate (Sigma-Aldrich) containing 100 5 00 ng/ml DIG-labeled RNA probes. After hybridization, sections were washed three times in a solution containing 50% formamide 1× SSC (Ambion) and 0.1% Tween 20 (Sigma-Aldrich) at 65C, and two times at RT in 1× MABT (20 mM maleic acid, 30 mM NaCl, 0.1% Tween 20; Sigma-Aldrich) before incubating in a solution containing 2% blocking reagent (Roche) and 10% normal goat serum (NGS) in MABT, followed by overnight incubation in alkaline phosphatase-conjugated anti-DIG antibody (1:1500; Roche). Nitroblu-etetrazolium chloride (Roche)/5-bromo-4-chloro-3-indolyl phosphate (Roche) diluted 1:1000 in MABT with 5% polyvinyl alcohol (VWR International) was used for colorimetric detection for 6 h. Sections were mounted using Glycergel mounting medium (Dako). Specificity of the procedure was assessed with probes corresponding to the sense strand of *CXCL12*, *CXCR4*, and *CXCR7*. The sense probes produced only slight signals that were clearly distinct from the signals of the antisense probes.

Histochemistry, antibodies, and immunohistochemistry. Mouse embryos were fixed in Bouin/Hollande fixative for 16 h (E12.5E 14.5) or 24 h (E16.5) and subjected to routine paraffin embedding (Stumm et al., 2001). Sections were cut at 8 μm for immunohistochemistry, and labeling was performed as described previously (Stumm et al., 2007). Briefly, endogenous peroxidase was blocked with methanol/H_2O_2 after deparaffinization. For antigen retrieval, sections were bathed for 25 min in 0.01 M citrate buffer, pH 6.0, at 95 C. Sections were then blocked in 50 mM PBS, containing 5% bovine serum albumin (BSA), and incubated overnight with primary antibody in 50 mM PBS containing 1% BSA. The following primary antibodies were used: UMB-2 rabbit monoclonal anti-CXCR4 (1:1000; Fischer et al., 2008), rabbit anti-GnRH (1:200 for fluorescence; 1:1000 for DAB; Immunostar), chicken anti-GFP (1:500; Aves), rabbit anti-peripherin (1:500 for fluorescence; 1:1000 for diaminobenzidine (DAB); Millipore), mouse anti-βIII-tubulin (1:500; Sigma-Aldrich), rabbit anti-cleaved caspace-3 (1:5000; Cell Signaling Technology), and rabbit anti-RFP (1:300; Abcam). For dual fluorescence, primary antibodies from different species were combined. TSA enhancement (Vector ABC kit; Vector Laboratories) was performed for UMB-2 before detection with AF555 and AF488 (Invitrogen). Double fluorescence immunohistochemistry for UMB-2 and GnRH (both rabbit) was performed by staining first for UMB-2 as aforementioned and, following washes in a series of ethanol (50% to 100% and back to 50%) and PBS, sections were blocked again in PBS/BSA for 1 h and incubated overnight in anti-GnRH antibody (1:100). Subsequent detection of UMB-2 was performed using streptavidin AF488 (1:200) and localization of GnRH with Alexa 568 (1:200, Invitrogen). Controls were used to ensure that secondary antibodies (Alexa 568) did not bind to UMB-2. Nuclei were counterstained with DAPI (Sigma-Aldrich). Immunofluorescence images were captured with a LSM510 confocal microscope (Zeiss).

For quantification of GnRH neurons, embryonic heads (E12.5E 16.5) were fixed in PFA, made in PBS, for 4 8 h at RT. After fixation, tissue was cryoprotected in 30% sucrose in PBS, embedded and frozen in Tissue-Tek OCT (Sakura Finetek), and sectioned sagittally at 20 μm using a cryostat (Bright Instruments). Sections were washed in PBS and incubated with H_2O_2 to quench endogenous peroxidase activity. They were then blocked in PBS, containing 5% NGS (Sigma-Aldrich) (v/v) and 0.1% Triton X-100 (v/v) (Sigma-Aldrich), at RT for 2 h. They were subsequently incubated in anti-GnRH (1:1000) antibody at RT overnight, followed by washes in PBS, and incubated in biotinylated anti-rabbit (1:200; Vector Laboratories) for 2 h. Immunoreactivity was visualized with the ABC kit (Vector Laboratories) followed by DAB (Sigma-Aldrich) development. For each genotype, we analyzed at least three embryos at each age, and all GnRH-positive cells were counted in every section of each head under a 40× microscope objective. For all experiments, data were expressed as mean ± SEM. To determine statistical significance, we used unpaired Students *t* test, with $p < 0.05$ considered to be statistically significant.

CXCR4-immunoreactivity was quantified in UMB-2-stained coronal head sections of *CXCR7*$^{-/-}$, *CXCL12*$^{-/-}$;*CXCR7*$^{-/-}$, and homozygous or heterozygous control littermates. For quantification, the nose region was photographed under identical imaging conditions with an Axio Imager A1 microscope (Zeiss) connected to a ProgRes C5 camera (Jenoptik). The sensory epithelium of the vomeronasal organ (VNO) and the olfactory epithelium (OE) were defined as areas of interest using ImageJ software. The proportion of the stained area at the region of interest was measured after setting a uniform threshold level. Measurements were expressed as percentage of the control group.

Results

CXCR7 is expressed in the nasal area

We first analyzed the spatiotemporal profile of *CXCR7* expression in the developing nasal area from early to late embryonic life,

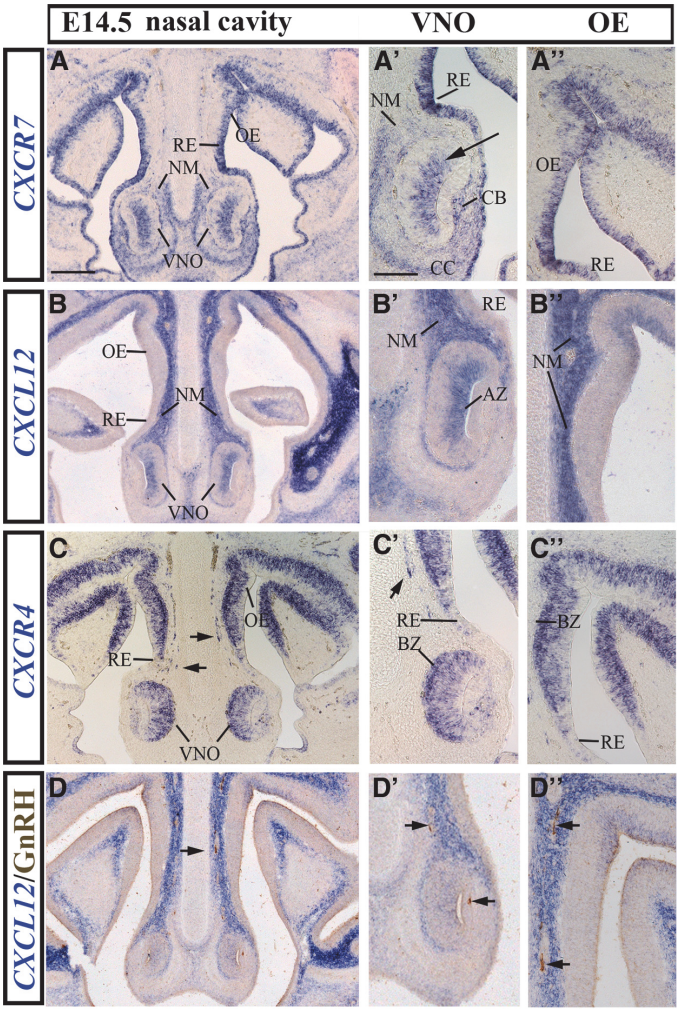

Figure 1. Expression of *CXCL12* and its receptors, *CXCR7* and *CXCR4*, in the nasal area of E14.5 mice as revealed by *in situ* hybridization. ***A***, Low-magnification image of a coronal head section at the level of the nose reveals *CXCR7* expression in the VNO, NM, RE, and OE. ***A′***, ***A″***, Higher magnification views show that *CXCR7* is predominantly expressed in the apical zone of the sensory epithelium of VNO (arrow) and OE. ***B***, *CXCL12* is expressed in the VNO and NM. ***A′***, ***B′***, *CXCL12* and *CXCR7* overlap in the apical zone (AZ) of the VNO and the NM dorsomedial to the VNO. ***B″***, Higher magnification of the OE area where *CXCL12* is highly expressed in the NM and moderately expressed in the dorsal part of the sensory epithelium. Note that most of the OE is *CXCL12* negative. ***C***, *CXCR4* is highly expressed in the sensory epithelium of VNO and OE and sparsely expressed in the RE. Presumed migrating GnRH neurons are *CXCR4* positive (arrows). ***C′***, ***C″***, Higher magnification images illustrate predominant *CXCR4* expression in the basal zone (BZ) of VNO (***C′***) and OE (***C″***). ***D***, ***D′***, *In situ* hybridization for *CXCL12* and immunohistochemistry for GnRH illustrate migrating GnRH neurons (arrows) on *CXCL12* expressing substrate in the NM. ***D′***, ***D″***, These neurons are shown in higher magnification in the area of the VNO (***D′***) and OE (***D″***). Scale bars: (in ***A***) ***A–D***, 200 μm; in (***A′***) ***A′–D″***, 100 μm. CB, cavernous body; CC, cartilaginous capsule.

and compared its pattern of expression to that of *CXCR4* and their agonist, *CXCL12*. Analysis was performed in embryos from E12.5 to E16.5, a period that covers the early stage of migration (E12.5) of GnRH neurons, following their emergence from the nasal placode at E10.5 (Schwanzel Fukuda and Pfaff, 1989), and the migration between the nasal compartment and the basal fore brain (E14.5E 16.5).

As we observed only minor differences in the overall patterns of the three genes between E12.5 and E14.5, we shall only provide details of their expression at E14.5 (Fig. 1). At this age, *CXCR7* was strongly expressed in the VNO and the surrounding area (Fig. 1*A*,*A′*). Specifically, *CXCR7* mRNA was detected in the sensory epithelium of the VNO (Fig. 1*A′*, arrow), the surrounding cartilaginous capsule, the cavernous body adjacent to the VNO, and the NM dorsomedial to the VNO (Fig. 1*A′*). *CXCL12* expression was found to overlap with *CXCR7* in the apical zone of the VNO (Fig. 1*A′*,*B′*). *CXCR4* was present throughout the VNO with the most prominent signal being localized in the basal zone (Fig. 1*C′*). Cells in the nasal area that appeared to be migrating were also *CXCR4* positive (Fig. 1*C*,*C′*, arrows) and resembled GnRH neurons as previously reported by Schwarting et al. (2006). The respiratory epithelium (RE), which is located lateral to the VNO in the ventral part of the nasal cavity, strongly expressed *CXCR7* (Fig. 1*A*,*A′*), but contained only few *CXCR4* positive cells (Fig. 1*C*,*C′*) and no *CXCL12* signal (Fig. 1*B*,*B′*). More dorsally, in the OE, both *CXCR7* and *CXCR4* were detected (Fig. 1*A″*,*C″*). *CXCL12* was absent in the OE except a small area in the dorsal most part of the nasal cavity (Fig. 1*B″*).

In agreement with a previous report (Schwarting et al., 2006), *CXCL12* was prominently expressed in the NM (Fig. 1*BB ″*). Dual labeling for *CXCL12* and GnRH showed that the *CXCL12* positive NM corresponds to the migration corridor of GnRH neurons (Fig. 1*DD ″*, arrows). Interestingly, *CXCR7* was also detected in the NM (Fig. 1*A*,*A′*) but, unlike *CXCL12*, it was concentrated ventrally (dorsomedial to the VNO) and expressed weakly in the dorsal NM.

CXCR7 is not expressed in migrating GnRH neurons and their guiding axons

It has been established that CXCR7 expressed in non migrating cells along CXCL12 positive migration paths can modulate CXCL12/CXCR4 guided cell migration (Boldajipour et al., 2008). Recent reports suggested that CXCR7 expressed in migrating neurons can directly influence the migration process (Sanchez Alcaniz et al., 2011; Wang et al., 2011). We thus wondered if CXCR7 modulates GnRH neuron migration in a direct or indirect manner and sought to determine the identity of *CXCR7* and *CXCR4* expressing cells in the nasal area. In these experiments, we used BAC transgenic mice carrying eGFP under the control of *CXCR7* and *CXCR4* promoters in double immunofluorescence for GnRH/eGFP. We found that the overall expression patterns of *CXCR7* and *CXCR4* eGFP (Fig. 2*A*,*D G*) matched the distribution of the respective mRNAs (Fig. 1*A*,*C*). However, in the NM, strong *CXCR7* eGFP signal was present

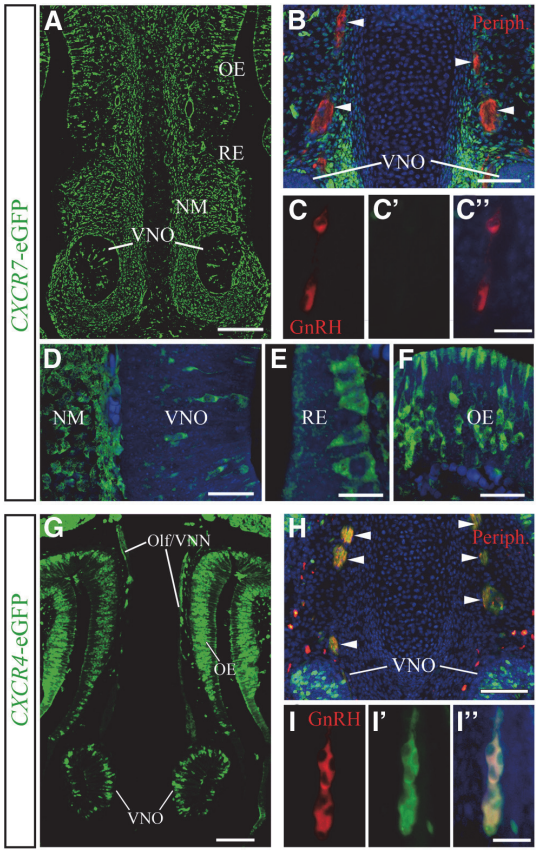

Figure 2. GnRH neurons and their axonal scaffold do not express *CXCR7*, but do express *CXCR4*. ***A***, ***G***, Coronal head sections of E14.5 transgenic *CXCR7*- and *CXCR4*-eGFP mice reveal the relative expression patterns of *CXCR7* and *CXCR4* in the nasal area. ***B***, ***H***, Olf/VNN axons (arrowheads) used by migrating GnRH neurons and stained for peripherin (red) are *CXCR7* negative (***B***), but *CXCR4* positive (***H***). ***C***, ***I***, Dual immunofluorescence for GnRH and eGFP demonstrates that GnRH neurons are eGFP negative in *CXCR7*-eGFP mice (***C–C″***) and eGFP positive in *CXCR4*-eGFP mice (***I–I″***) indicating that GnRH neurons express *CXCR4*, but not *CXCR7*. ***D–F***, High-magnification images reveal *CXCR7*-eGFP in the VNO area (***D***), RE (***E***), and OE (***F***) of E14.5 mice. Specimens shown in ***B–F***, ***H***, and ***I*** were counterstained with DAPI. ***A–I***, All images were acquired by confocal microscopy. Scale bars: ***A***, 140 μm; ***B***, 50 μm; (in ***C″***) ***C–C″***, 50 μm; ***D***, 30 μm; ***E***, 14 μm; ***F***, 30 μm; ***G***, 100 μm; ***H***, 100 μm; (in ***I″***) ***I–I″***, 50 μm. Olf/VNN, olfactory/vomeronasal nerve; Periph, peripherin.

along the dorsoventral extent (Fig. 2*A*), whereas strong *CXCR7* mRNA signal was restricted to the ventral mesenchyme surrounding the VNO (Fig. 1*A*). This difference could be due to stability of eGFP, which permits accumulation of the reporter protein despite low gene expression. Nevertheless, GnRH neurons showed no colocalization of eGFP in *CXCR7* eGFP mice at the three ages examined (E12.5, E14.5, and E16.5; Fig. 2*CC″*), indicating that GnRH neurons do not express *CXCR7* during their migration. Since olfactory/vomeronasal axons serve as guides to migrating GnRH neurons, we tested whether *CXCR7* is present in this axonal scaffold by staining sections through the nasal compartment of mice at E12.5E 16.5 for peripherin. These experiments indicated that peripherin labeled axons were also negative for *CXCR7* eGFP (Fig. 2*B*, arrowheads). In similar experiments, using *CXCR4* eGFP mice, we confirmed that, in contrast to *CXCR7*, the majority of GnRH neurons (Schwarting et al., 2006; Fig. 2*II″*) and their guiding axons (Toba et al., 2008; Fig. 2*H*, arrowheads) express *CXCR4* at all ages examined.

CXCR7 regulates CXCL12 availability and CXCR4 activation in the nasal area

If CXCR7 is not present in GnRH neurons and their scaffold, could it exert an indirect effect on their CXCL12/CXCR4 guided migration? To approach this question, we sought to establish whether CXCR7 influences CXCL12 availability and CXCR4 activation in the nasal area. Since chemokine receptors internalize and accumulate their bound ligands in intracellular vesicles, identification of RFP labeled cells in transgenic mice expressing RFP labeled chemokine proved useful to reveal sites of chemokine/chemokine receptor interaction (Jung et al., 2009). We examined the nasal area of BAC transgenic mice expressing an RFP fused version of CXCL12 (CXCL12 RFP) under the control of the *CXCL12* promoter (Bhattacharyya et al., 2008), and found that *RFP* transcripts (data not shown) exactly recapitulated the pattern of *CXCL12* mRNA in wild types (Fig. 1*BB″*), whereas CXCL12 RFP protein (Fig. 3*A*) did not. Specifically, the NM dorsomedial to the VNO showed matching strong *CXCL12* mRNA and robust RFP expression (Figs. 1*B′*, 3*A*,*D*). The VNO, however, which showed moderate expression of *CXCL12* mRNA (Fig. 1*B′*), contained low RFP signal (Fig. 3*D*). The most striking mismatch between *CXCL12* expression and RFP was observed in the RE, which showed very strong RFP signal (Fig. 3*A*,*E*) despite being *CXCL12* mRNA negative (Fig. 1*B*). Most of the OE was sparsely RFP positive (Fig. 3*A*,*F*) and *CXCL12* mRNA negative (Fig. 1*B*,*B″*; a small dorsal area moderately expressed *CXCL12* and contained sparse RFP). In *CXCR7* eGFP;CXCL12 RFP bitransgenic mice, we observed that the strongly RFP positive NM and the RE (data not shown) were eGFP positive. High power confocal analysis confirmed that RFP signal was often contained within *CXCR7* eGFP positive cells (Fig. 3*C*, arrows), suggesting that the signal could, at least in part, result from CXCR7 mediated CXCL12 RFP accumulation.

We then generated CXCL12 RFP transgenic *CXCR7* deficient (CXCL12 RFP;*CXCR7*$^{-/-}$) mice to test this assumption, and found that the pattern of CXCL12 RFP was severely altered in the absence of CXCR7. The most obvious difference was noted in the RE (Fig. 3*E*,*H*), which normally expresses *CXCR7* and contains strong RFP signal. In CXCL12 RFP;*CXCR7*$^{-/-}$ animals, this structure was virtually RFP negative (Fig. 3*H*). Since the RE does not express *CXCL12* and contains only few *CXCR4* positive cells, this observation suggests that CXCR7 normally accumulates/sequesters RFP in this tissue. Also in the NM dorsomedial to the VNO, the RFP signal was severely reduced in the absence of CXCR7 (Fig. 3*D*,*G*). The residual RFP signal most likely reflects the strong CXCL12 RFP expression. Interestingly, VNO (Fig. 3*D*,*G*) and OE (Fig. 3*F*,*I*) showed increased RFP labeling in CXCL12 RFP/*CXCR7*$^{-/-}$ mice. Given that both structures highly express CXCR4 (Fig. 1*C C″*), the increased RFP labeling might be due to enhanced CXCL12 RFP/CXCR4 interactions. Collectively, our analysis indicates that CXCL12 RFP preferentially accumulates at sites expressing CXCR7. In the absence of CXCR7, CXCL12 RFP accumulates in CXCR4 expressing structures.

If absence of CXCR7 does lead to increased availability of CXCL12, it would increase CXCL12/CXCR4 interaction, promote CXCR4 internalization, and, thus, alter the subcellular distribution of CXCR4. Careful analysis of the CXCR4 staining in the VNO (Fig. 4*D*,*E*) and OE (Fig. 4*K*) in *CXCR7*$^{-/-}$ mice re

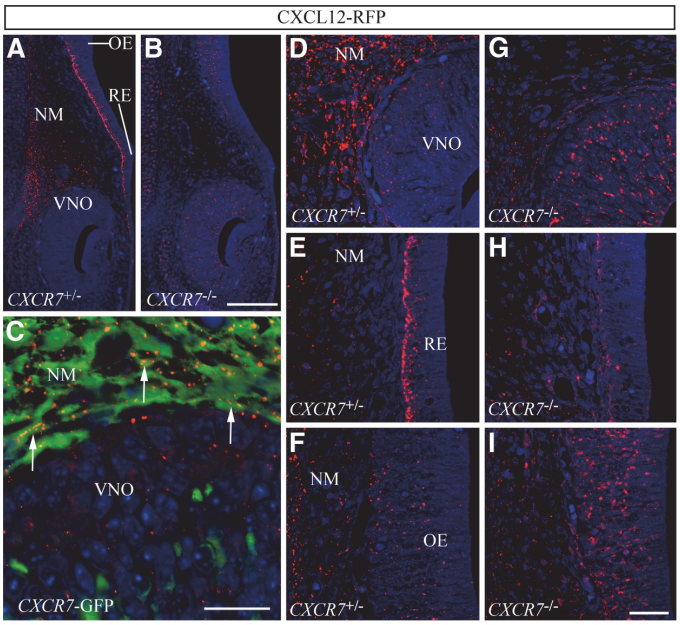

Figure 3. CXCR7 regulates spatial distribution of CXCL12-RFP along the migration route of GnRH neurons. ***A–I***, Immunostaining for RFP in coronal sections of the nasal area of E14.5 transgenic mice expressing CXCL12-RFP fusion protein. ***A***, ***B***, Low-magnification confocal images reveal strongly decreased RFP signal in the NM and RE and increased RFP signal in the VNO and OE of a CXCL12-RFP;*CXCR7*$^{-/-}$ embryo (***B***) as compared with a CXCL12-RFP;*CXCR7*$^{+/-}$ littermate (***A***). ***D–I***, High-magnification confocal views of VNO and adjacent NM (***D***, ***G***), RE (***E***, ***H***), and OE (***F***, ***I***). ***C***, High-magnification confocal view of a dual immunofluorescence for RFP and eGFP in a CXCL12-RFP;*CXCR7*-eGFP bitransgenic mouse shows RFP signal in eGFP-positive mesenchymal cells (arrows). Scale bars: (in ***B***) ***A***, ***B***, 50 μm; ***C***, 14 μm; (in ***I***) ***D–I***, 14 μm.

vealed the intracellular accumulation of reaction product (dots), suggesting increased internalization or allocation of the receptor in the cytoplasm of cells. In wild-types, most of CXCR4 was present at the surface and only a fraction appeared internalized in cells of the VNO (Fig. 4*B*) and OE (Fig. 4*J*). Quantitative analysis of the CXCR4-immunoreactive area in OE and VNO confirmed that CXCR4 staining was severely reduced in *CXCR7*$^{-/-}$ mice (Fig. 4*M*, middle). Double immunohistochemistry with CXCR4 and βIII-tubulin, which labels both neuronal somata and processes, revealed absence of CXCR4 from the olfactory/vomeronasal axons in *CXCR7*$^{-/-}$ mice (Fig. 4*D*,*E*, arrowheads). Having shown that lack of CXCR7 affects the spatial distribution of CXCL12-RFP and CXCR4 throughout the nasal area, we turned to GnRH neurons and found that CXCR4 immunoreactivity was also reduced or even absent when compared with controls (Fig. 4*C C*″,*F F*″). The possibility, that the altered pattern of CXCR4 in the nasal area is due to altered CXCR4 mRNA expression, was ruled out by *in situ* hybridization for *CXCR4*, which gave similar results in *CXCR7*$^{-/-}$ mice and control littermates (data not shown).

To assess whether altered subcellular distribution of CXCR4 and reduced CXCR4 staining in the nasal area of *CXCR7*$^{-/-}$ mice depends on CXCL12, we generated *CXCL12*$^{-/-}$;CXCR7$^{-/-}$ mice. We found that absence of CXCL12 resulted in upregulation of CXCR4 on the plasma membrane and increased CXCR4 immunoreactivity in cells of the VNO and OE (Fig. 4*G*,*H*,*L*,*M*, right) as compared with controls. Double immunofluorescence for CXCR4/βIII-tubulin and CXCR4/GnRH revealed high levels of CXCR4 in vomeronasal axons (Fig. 4*G*,*H*, arrowheads) and targeting of CXCR4 to the plasma membrane of GnRH neurons (Fig. 4*I I* ″). Thus, loss of CXCR4 from the surface of cells in the nasal area observed in *CXCR7*-deficient mice, a typical response to high levels of CXCL12, was reversed in an experiment that combined removal of both CXCL12 and CXCR7.

CXCR7-deficient mice exhibit impaired GnRH neuron migration

Given our findings suggest that CXCR7 controls CXCL12 availability in the nasal area and CXCL12/CXCR4 interactions in GnRH neurons, we investigated whether its loss might impact on GnRH neuron migration. For this purpose, the number and distribution of GnRH neurons in sagittal sections through the nose and forebrain of *CXCR7*$^{-/-}$ mice were compared with control littermates at three developmental stages (E12.5, E14.5, and E16.5). Regional analysis revealed an increase in the number of GnRH neurons in the nasal compartment and a concomitant decrease in the forebrain of the *CXCR7*$^{-/-}$ mice compared with controls at all ages examined (Fig. 5*C*,*F*,*I*). In detail, at E12.5, there was no significant difference between the total GnRH neuron population of *CXCR7*$^{-/-}$ mice and controls (knockout (KO): 1346 $\pm$ 64 as compared with control: 1289 $\pm$ 92, $p = 0.64$; $n = 3$; Fig. 5*C*), implying that the production of these neurons is not affected by the absence of the receptor. Nonetheless, *CXCR7*$^{-/-}$ mice exhibited a significant increase in GnRH cell numbers in the nasal compartment (with cells accumulating around the VNO; Fig. 5*B*, arrowhead) as compared with controls (Fig. 5*A*, arrow) (KO: 1017 $\pm$ 42, control: 754 $\pm$ 68, $^{*}p = 0.031 < 0.05$; Fig. 5*C*) and a decrease in the forebrain (Fig. 5*B*) (KO: 329 $\pm$ 23, control: 535 $\pm$ 29, $^{**}p = 0.0052 < 0.01$; Fig. 5*C*). This finding suggests a role for CXCR7 in the early steps of GnRH neuron migration, when these cells exit the VNO and start migrating toward the forebrain. At E14.5, when >50% of GnRH neurons in control animals have already passed the cribriform plate and are directed toward the forebrain (Fig. 5*D*), the equivalent proportion of neurons in *CXCR7*-deficient mice is 34% (KO: 368 $\pm$ 37 as compared with control: 714 $\pm$ 50, $^{**}p = 0.001$, $n = 6$; Fig. 5*F*) with many GnRH cells remaining in the nasal compartment (Fig. 5*E*, arrow) (KO: 793 $\pm$ 24, control: 637 $\pm$ 33, $^{**}p = 0.009 < 0.01$; Fig. 5*F*). Strikingly, GnRH neurons in *CXCR7*-deficient mice often formed large multicellular clusters, similar to those previously reported in GNR23 mice carrying a deletion in *Epha5* gene (Gamble et al., 2005; Fig. 5*M*), whereas they migrated in chain-like formations in wild-types (Fig. 5*G*). Further, many cells were found abnormally in the nasal epithelium (Fig. 5*H*,*L*, arrowheads) and a small number appeared in the VNO lumen (Fig. 5*L*, arrow), where they eventually die (Fig. 5*N*, arrowheads), indicating misguided migration. Still at this stage (E14.5), the total GnRH cell number did not differ significantly between the two groups of animals, although there was a slight reduction in the mutants (KO: 1161 $\pm$ 32, control: 1351 $\pm$ 69, $p = 0.069 > 0.05$; Fig. 5*F*). To determine the fate of the cells that accumulate in the

nose, we analyzed *CXCR7*-null mice of E16.5. By this age, the majority of GnRH neurons in control mice have migrated to the brain, whereas in *CXCR7*-null animals most GnRH neurons remained in the nasal area (KO: 500 ± 34, control: 298 ± 22, $^{***}p = 0.0001 < 0.001$; $n = 3$; Fig. 5*I*) and only a small percentage (~37%), similar to that observed at E14.5, had passed the cribriform plate (KO: 289 ± 26 as compared with control: 837 ± 21, $^{***}p = 3.21\text{E-}09 < 0.001$; Fig. 5*I*). Interestingly, the mean total number of GnRH neurons in *CXCR7* $^{-/-}$ mice of this later age appeared significantly reduced when compared with control animals (KO: 789 ± 57, control: 1135 ± 30, $^{***}p = 0.0004 < 0.001$; Fig. 5*I*), implying that abnormally accumulating GnRH neurons in the nose could have been eliminated by programmed cell death.

We next examined the patterning of olfactory/vomeronasal axons stained for peripherin and confirmed that, in *CXCR7*-null mice, the axonal substrates used by migrating GnRH neurons were normally fasciculated and properly positioned (Fig. 5*J*,*K*), suggesting that *CXCR7* is not involved in the patterning/targeting of these axons.

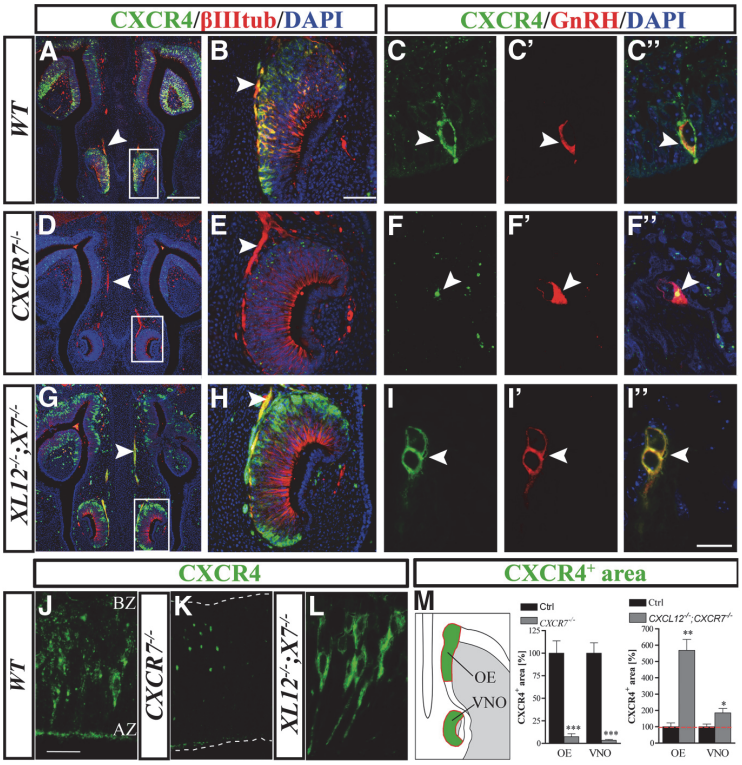

Figure 4. Subcellular redistribution and loss of *CXCR4* in the nasal area of *CXCR7* $^{-/-}$ mice. ***A–M***, Immunostained frontal sections of E14.5 mice. ***A***, ***B***, Double labeling for CXCR4 (green) and βIII tubulin (red) shows that in a *WT* animal, CXCR4 is localized in axons (arrowheads) and in cells in the VNO (***B***). ***D***, ***E***, CXCR4 is not detectable in axons (arrowheads) and shows a punctate/internalized pattern in the VNO of a *CXCR7* $^{-/-}$ animal (***E***). ***G***, ***H***, The loss of CXCR4 observed after *CXCR7* ablation (***D***, ***E***) is reversed in a *CXCL12* $^{-/-}$;*CXCR7* $^{-/-}$ animal (*XL12* $^{-/-}$;*X7* $^{-/-}$). ***C***, ***F***, ***I***, Similar changes in CXCR4 localization were noted in GnRH neurons. For example, in one of these neurons of a WT animal (***C–C″***, arrowhead), CXCR4 is clearly expressed on the cell membrane. However, in a GnRH neuron of *CXCR7* $^{-/-}$ mutant, the receptor is localized in the cytoplasm and not detected on the plasma membrane (***F–F″***, arrowhead). In the absence of CXCL12, CXCR4 is upregulated on the cell membrane and intracellular clusters of CXCR4 are not observed in *CXCR7*-deficient GnRH neurons (***I–I″***, arrowhead). ***J–L***, High-magnification views demonstrate altered subcellular distribution of CXCR4 in the OE of a *CXCR7* $^{-/-}$ mutant (***K***) and a *CXCL12* $^{-/-}$;*CXCR7* $^{-/-}$ double mutant (***L***) as compared with a WT control (***J***). ***A–L***, All images were acquired by confocal microscopy. ***M***, Quantification of the CXCR4-immunoreactive area in the OE and VNO of *CXCR7* $^{-/-}$ and *CXCL12* $^{-/-}$;*CXCR7* $^{-/-}$ mice and their control littermates ($n = 4$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ANOVA. Green areas in the schematic represent the regions of interest used for quantification. The nasal septum is to the left and the nasal cavity (shaded) to the right. Scale bars: (in ***A***) ***A***, ***D***, ***G***, 200 μm; (in ***B***) ***B***, ***E***, ***H***, 50 μm; (in ***I″***) ***C***, ***F***, ***I***, 20 μm; (in ***J***) ***J–L***, 20 μm. WT, wild-type.

Comparative analysis of *CXCL12*-, *CXCR4*-, and *CXCR7*-null mice

Previous studies have demonstrated that *CXCR4*-deficient mice display decreased GnRH cell number and impaired migration (Schwarting et al., 2006; Toba et al., 2008). However, comparable analysis of mice lacking the ligand, CXCL12, has not been performed to date. After CXCR7 was identified as a second CXCL12 receptor (Balabanian et al., 2005), we reasoned that examination of *CXCL12*-null mice in comparison with animals lacking *CXCR4* or *CXCR7* would shed light on the role of each of these molecules in the development of the GnRH neuron system and enhance our knowledge of the mechanisms that regulate this receptor system.

Thus, we analyzed *CXCL12* defective mice at E14.5 and found a gross decrease in the total number of GnRH neurons when compared with control littermates (KO: 480 ± 25, control: 1187 ± 16; $^{***}p = 1.98\text{E-}05 < 0.0001$; $n = 3$; Fig. 6*K*). We then re-examined *CXCR4* $^{-/-}$ mice at the same age and, in agreement with previous studies (Schwarting et al., 2006), found reduced total GnRH cell number (KO: 628 ± 12, control: 1227 ± 25; $^{***}p = 2.96\text{E-}05$; $n = 3$; Fig. 6*L*).

As *CXCR7* expression was shown to be absent from GnRH neurons, at least within the time frame of our analysis (E12.5 16.5; KO mice die in late gestation), we considered CXCR4 to be the only receptor mediating CXCL12 signaling on GnRH neurons. The similarity between the two phenotypes (significant decrease in the total number of GnRH neurons in *CXCR4* $^{-/-}$ and *CXCL12* $^{-/-}$ mice), together with our finding that *CXCR7* $^{-/-}$ mice do not display such defect (Fig. 6*M*), provide credence to this hypothesis.

In addition to the defect in total cell number in both *CXCR4* and *CXCL12*-null mice, the regional distribution of GnRH neurons differed from that of control animals. Specifically, similar to embryos lacking *CXCR7* (Fig. 6*D*,*I*,*M*), GnRH neurons in *CXCL12*-deficient mice were found accumulated in the nasal compartment (area between the VNO and nasal/forebrain junction) and not migrating to the brain (Fig. 6*B*,*G*). Thus, at E14.5, there were twice as many neurons in the nose compared with the brain when, in control mice, most of GnRH neurons had already crossed the nasal/forebrain junction and were directed toward the hypothalamus. Counts of cells residing in the forebrain revealed that control animals had five times as many GnRH neurons as *CXCL12* $^{-/-}$ mice (KO: 148 ± 3, control: 701 ± 9; $^{***}p <$

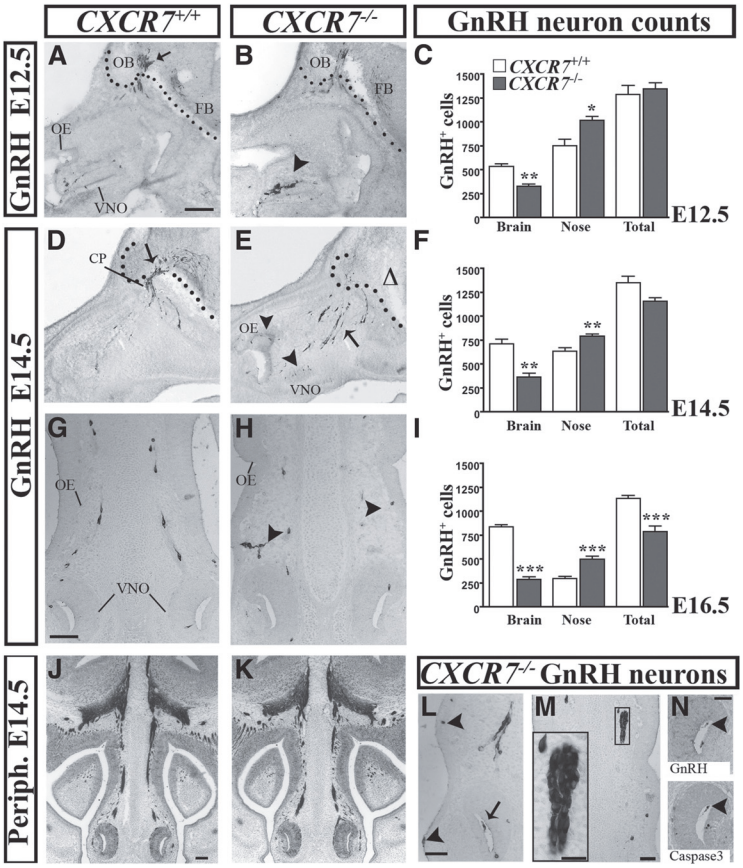

Figure 5. Migration of GnRH neurons is altered in *CXCR7*-null mice. Sagittal sections (***A***, ***B***) through the nose at E12.5, stained for GnRH, show that cells tend to remain in the area of the VNO (***B***, arrowhead) in *CXCR7* $^{-/-}$ mice whereas, in the corresponding controls, they appear to cross the cribriform plate (arrow) and migrate toward the forebrain (FB). Black dots separate the nasal area from the brain. ***D***, ***E***, A similar picture is evident at 14.5, with most GnRH neurons still migrating in the nasal area in *CXCR7*-null mice (***E***, arrow). Additionally, some cells are still present in the VNO and, abnormally, in the OE (*E*, arrowheads). The relative absence of GnRH neurons in the forebrain is indicated with a triangle. ***G***, ***H***, Coronal sections stained for GnRH reveal that, in *CXCR7* $^{-/-}$ mice, migrating cells deviate from their normal chain-like formation (***G***) and, instead, appear in the OE and RE (arrowheads in ***H*** and ***L***). Further, they form abnormal multicellular clusters in the NM (rectangle in ***M*** and at higher magnification in the inset in ***M***) and, sometimes, in the lumen of the VNO (***L***, arrow). ***N***, GnRH cells in the lumen of the VNO (arrowhead) show cleaved caspace-3 in a contiguous section, suggesting they are destined to die. ***J***, ***K***, However, guiding axons stained for peripherin appear similar in both genotypes. ***C***, ***F***, ***I***, Compartmental quantification of GnRH cells in the three ages examined showed significant increase of neurons in the nose and a concomitant decrease in the brain of *CXCR7* $^{-/-}$ mice compared with WT. Moreover, while the total number of cells was similar in the two groups at E12.5 and E14.5, it declined in the KO mice at E16.5 (***I***). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; Scale bars: (in ***A***) ***A***, ***B***, 150 μm; ***D***, ***E***, ***G***, ***H***, ***L***, ***M***, 50 μm; (in ***J***) ***J***, ***K***, 500 μm; ***N***, 30 μm. CP, cribriform plate; OB, olfactory bulb.

0.0001; Fig. 6*F*,*G*,*K*). Similarly, in *CXCR4* lacking mice, the majority (60%) of the cells were still present in the nasal compartment as compared with 42% in control littermates. Concomitantly, the number of GnRH neurons in the brain differed significantly from control mice (KO: 254 ± 17, control: 710 ± 36; $^{***}p = 0.0003$; Fig. 6*C*,*H*,*L*).

To gain greater insight on why GnRH neurons remain in the nasal compartment, we carefully examined their appearance and spatial distribution in this area. As depicted in Fig. 6*N*, considerably more cells in these animals were found abnormally in the OE (Fig. 6*B*,*D*, arrowheads). Specifically, when we analyzed the positions of GnRH neurons in the three groups (*CXCL12*, *CXCR4*, and *CXCR7*) of KO mice, we observed that in *CXCL12* and *CXCR7* deficient mice, 22 and 28% of the total GnRH cell population present in the nose was aberrantly located in the epithelium; in the *CXCR4* $^{-/-}$ mutant, that percentage was 8.5%. In comparison, hardly any (0 2 %) cells were identified in the OE in all control littermates. These observations strongly suggest that this chemokine/receptor system keeps GnRH neurons to their migratory path. We should add that we found no apparent defect in the peripherin stained vomeronasal axons of *CXCL12* $^{-/-}$ and *CXCR4* $^{-/-}$ mice (data not shown).

Discussion

The migration of GnRH neurons represents a unique example of neuronal migration as these cells are born outside the brain and follow a long and tortuous route to their destinations in the forebrain. Chemokines expressed along migration corridors have been reported to serve as guidance cues for migrating neurons (Borrell and Mar n, 2006; Stumm et al., 2007). Accordingly, CXCL12 has been found to be expressed in the NM and to guide migrating GnRH neurons toward the forebrain (Schwarting et al., 2006; Toba et al., 2008).

CXCL12 exerts its functions by binding to CXCR4. In the developing brain, CXCL12 and its receptor CXCR4 are highly expressed in the neocortex, cerebellum, and hippocampus, and regulate cell migration and axonal pathfinding (Ma et al., 1998; Chalasani et al., 2003; Tran and Miller, 2003). Although the *CXCR4* gene is downregulated in most brain regions postnatally, persistent expression in the hypothalamus and CXCL12/CXCR4 mediated modulation of hypothalamo pituitary systems points to a role of this receptor in neuroendocrine regulation (Callewaere et al., 2006; Li and Ransohoff, 2008; Rostène et al., 2011).

GnRH neurons express CXCR4 early in their development, as they leave the VNO to begin their migration to the brain. *CXCR4* deficient mice exhibit impaired GnRH neuron migration and a significant decrease in their total number (Schwarting et al., 2006; Toba et al., 2008), pointing to the importance of CXCL12 signaling in this system. CXCR4 was long considered the only receptor for CXCL12, until it was shown recently that it binds with higher affinity to an additional receptor, CXCR7 (Balabanian et al., 2005). While signaling properties of CXCR7 are presently controversial (Thelen and Thelen, 2008; Luker et al., 2009; Rajagopal et al., 2010; Hoffmann et al., 2012; Kumar et al., 2012; Odemis et al., 2012), it appears to act as a CXCL12 scavenger controlling chemokine levels in the extracellular environment by ligand sequestration (Boldajipour et al., 2008; Naumann et al., 2010; Hoffmann et al.,

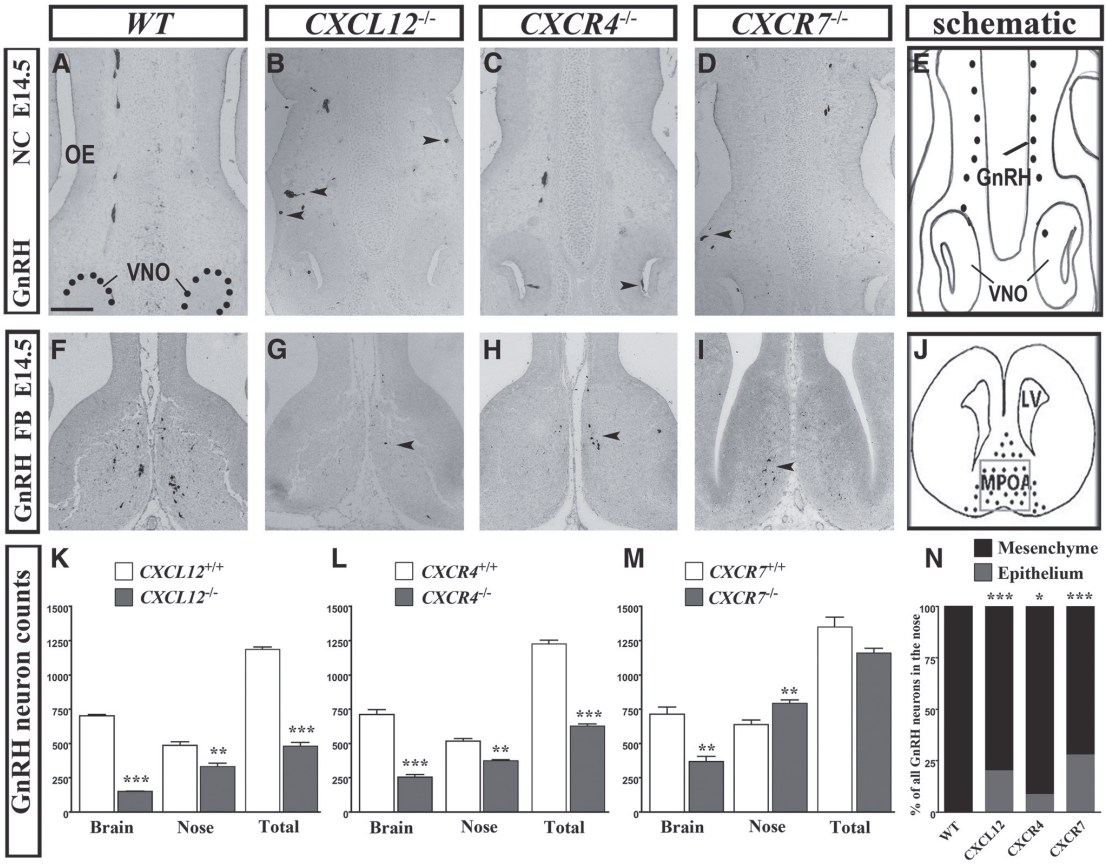

Figure 6. GnRH neuron migration in *CXCL12* $^{-/-}$, *CXCR4* $^{-/-}$, and *CXCR7* $^{-/-}$ mice at E14.5. ***A–D***, In the nose, GnRH neurons migrate in a chain-like formation in WT animals but, in KOs, they form clusters (***B***, arrowheads), and are often found abnormally in the lumen of the VNO (***C***, arrowhead) or in the epithelium (***D***, arrowhead). ***F–I***, GnRH staining of coronal sections at the level of the MPOA shows the distribution of these cells in *WT* animals (***F***) and the relative reduction in numbers in the *CXCL12* $^{-/-}$ (***G***), *CXCR4* $^{-/-}$ (***H***), and *CXCR7* $^{-/-}$ (***I***) mice. ***K–M***, Compartmental quantification of GnRH neurons shows an overall decrease in *CXCL12* $^{-/-}$ and *CXCR4* $^{-/-}$, but not in *CXCR7* $^{-/-}$ animals, and a significant reduction in both nose and brain when compared with control animals. ***N***, Spread/distribution analysis of GnRH neurons in the nasal area, divided in two compartments, epithelium and NM, show that 22% of all cells in the nasal area are abnormally localized in the epithelium of *CXCL12* $^{-/-}$ animals. The respective percentage is 28% in *CXCR7* $^{-/-}$ and 8.5% in *CXCR4* $^{-/-}$ mice. No GnRH neurons are found in the epithelium in control animals. ***E***, ***J***, Schematics show the nasal compartment (NC) and the medial preoptic area (MPOA) in the forebrain (FB) as photographed in ***A–D*** and ***F–I***, respectively. ***K–N***, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Scale bars: (in ***A***) ***A–D***, ***F–I***, 100 μm. FB, forebrain; LV, lateral ventricle.

2012). In this context, loss of CXCR7 leads to extracellular accu mulation and perturbed gradients of the chemokine (Boldaji pour et al., 2008; Sanchez Alcaniz et al., 2011). Similar to CXCL12 and CXCR4, CXCR7 is expressed in the developing he matopoietic system, cardiovascular system, and brain, and *CXCR7* deficient mice die perinatally with cardiovascular defects (Sierro et al., 2007).

Here, we provide evidence that CXCR7 is important in the early steps of GnRH neuron migration, as lack of this receptor results in aberrant accumulation of these cells in the nasal cavity. The defects are associated with severe reduction and perturbed subcellular distribution of CXCR4 protein in cells throughout the nasal area, including GnRH neurons.

CXCR7 expression in the nasal area

The first indication that CXCR7 may be involved in GnRH neu ron migration came from its expression at the start and along the well defined migratory path of these cells in the nose. Specifically, we identified *CXCR7* expression within and around the VNO, where GnRH neurons emerge. Its expression in the more dorsal parts of the NM was significantly reduced up to the cribriform plate, where GnRH neurons enter the brain. Given that CXCR7 sequesters CXCL12 from the extracellular environment (Bolda jipour et al., 2008; Naumann et al., 2010; Hoffmann et al., 2012), we may suggest that its pattern of expression may help form a ventrodorsally increasing chemokine gradient. An additional mechanism that could form a CXCL12 gradient is the ventrodor sally increasing *CXCL12* expression within the nasal area (Schwarting et al., 2006). Our observation that GnRH neurons as well as the supporting axons of the olfactory/vomeronasal nerve do not express *CXCR7*, is consistent with the established role of this receptor in shaping CXCL12 gradients. Interestingly, *CXCR7* was also expressed in the RE and OE, tissues that are normally avoided by migrating GnRH neurons. Although the RE does not express *CXCL12* mRNA, it contained strong RFP signal in trans genic mice expressing CXCL12 RFP fusion protein under the

CXCL12 promoter. The observation that the RE in CXCL12-RFP;*CXCR7*$^{-/-}$ mice was devoid of RFP lends support to the concept that CXCR7-expressing cells sequester CXCL12 in the nasal area.

Impaired migration and abnormal distribution of GnRH neurons in *CXCR7*-null mice

If shaping the CXCL12 gradient by CXCR7 is important for GnRH neuron migration, then this process would be affected in mice lacking the receptor. Indeed, our analysis of E12.5 and E14.5 *CXCR7*-deficient mice revealed a defect in GnRH neuron migration when, at the same time, the total cell number was unaffected. This suggests that CXCR7 is not involved in the early generation of GnRH neurons. In these null mice, half of the GnRH neuron population failed to migrate to the hypothalamus and, instead, distributed randomly in the nasal area, formed clusters, and positioned themselves ectopically in the OE, suggesting that CXCR7 plays an important role in their directed migration toward the forebrain. Interestingly, although the overall GnRH cell number did not differ from control littermates at early developmental stages (E12.5E 14.5), it did decline significantly at E16.5. It is likely that ectopic GnRH neurons that failed to migrate to the brain eventually die or cease to express the GnRH gene. The partial phenotype observed here may be attributed to the reported heterogeneous makeup of the GnRH neuron population (Giacobini et al., 2008; Jasoni et al., 2009; Forni et al., 2011), and abundant evidence clearly indicates that no single genetic mutation may prevent all GnRH neurons from reaching their destinations (Cariboni et al., 2007a, 2011; Schwarting et al., 2007; Giacobini et al., 2008; Messina et al., 2011). The impact of reduced GnRH neurons in reproduction has been assessed in lines of transgenic mice that survive to adulthood. These studies have invariably shown abnormal gonadal development and disrupted fertility (Cariboni et al., 2005, 2007a; Messina et al., 2011).

As the GnRH system has never been investigated in mice lacking *CXCL12*, we analyzed the number and distribution of GnRH neurons in these mice and compared their phenotype to that of *CXCR4*- and *CXCR7*-deficient animals. We found that the phenotype in *CXCL12*$^{-/-}$ mice closely resembled that of *CXCR4*$^{-/-}$ mice, both in terms of migration and overall decrease in cell number, strongly suggesting that CXCL12 signals through CXCR4 in this system to control GnRH neuron survival and migration. Moreover, the aberrant distribution of GnRH neurons in the nasal area of *CXCL12*, *CXCR4*, and *CXCR7* null mice points to a direct role for CXCL12 in guiding the migration of these cells, especially since the supporting axonal scaffold was unaffected in the three KO lines.

CXCR7 controls CXCL12 availability

Previous studies have shown that sustained excess of CXCL12 leads to desensitization and degradation of CXCR4 (Marchese and Benovic, 2001). In addition, CXCR7 has been shown to regulate CXCR4 function. This can be done either in a cell-autonomous manner, when it is coexpressed with CXCR4 in the same cell (Sanchez-Alcaniz et al., 2011), or in a noncell-autonomous manner, when it is expressed in the tissue surrounding CXCR4-positive cells (Boldajipour et al., 2008; Haege et al., 2012). Our present data support for the first time the latter hypothesis for migrating neurons and thus, are clearly distinct from earlier findings proposing a cell-autonomous function for CXCR7 in neuronal migration (Tiveron et al., 2010; Sanchez-Alcaniz et al., 2011; Wang et al., 2011).

GnRH neurons express CXCR4, but not CXCR7, during their migration through the nasal compartment, and are attracted by progressively higher levels of CXCL12 as they target the cribriform plate (Schwarting et al., 2006). In the case of compromised CXCR7 activity, we found clustering of CXCR4 in the intracellular compartment suggesting internalization of the receptor and, in some cases, loss of CXCR4 protein in GnRH neurons and in olfactory/vomeronasal cells and their axons. This finding, the function of CXCR7 as a scavenger of extracellular CXCL12 (Naumann et al., 2010; Cruz-Orengo et al., 2011) and our observation that, in absence of CXCR7, more CXCL12-RFP accumulates at CXCR4 sites, prompted us to suggest that CXCR4 re-allocation from the plasma membrane to the cytoplasm of neurons in the nasal compartment is an indicator for excessive CXCL12-promoted CXCR4 activation. Thus, if increased levels of CXCL12 in *CXCR7* null mice result in CXCR4 internalization, then concomitant removal of the ligand (and CXCR7) would prevent excessive internalization and loss of CXCR4. Indeed, the apparent intracellular clustering of CXCR4 in *CXCR7*-deficient mice was reversed in *CXCL12*$^{-/-}$;*CXCR7*$^{-/-}$ animals, strongly suggesting that the function of CXCR7 in this system is to control CXCL12 levels. It has been proposed that GnRH neurons switch off the CXCR4 pathway once they reach the forebrain (Toba et al., 2008). Here, we may suggest a rapid gene regulation-independent mechanism by which this may be achieved: GnRH neurons, while in the nasal compartment, migrate toward higher levels of CXCL12, retaining CXCR4 activity through CXCR7 function. When they reach the cribriform plate, which is rich in CXCL12, but almost devoid of CXCR7, CXCR4 starts to become highly internalized and degraded as a result of hyperactivation, rendering GnRH neurons no longer responsive to the effects of CXCL12. Once GnRH neurons enter the forebrain, they are subjected to the influences of other molecules as they negotiate their migration toward the hypothalamus.

In summary, our studies have shown that CXCR7 is required for GnRH neuron migration. Our data support a mechanism, whereby CXCR7 controls CXCR4 activity in migrating GnRH neurons by regulating spatial availability and possibly levels of CXCL12. It is pertinent to note that recent studies in zebrafish have implicated β-arrestin in this mechanism (Mahabaleshwar et al., 2012). It would be interesting to assess whether this process is conserved in mice, and whether mice with defects in β-arrestin/CXCR7 interaction exhibit a similar phenotype to *CXCR7* null animals.

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5 Diskussion

Das Chemokin CXCL12 vermittelt im Zusammenspiel mit seinen Rezeptoren CXCR4 und CXCR7 zahlreiche physiologische und pathophysiologische Prozesse. Die vorliegende kumulative Arbeit liefert dazu neue Einblicke in die Interaktion zwischen Ligand und Rezeptoren und wie hieraus verschiedene neuronale Entwicklungsprozesse gesteuert werden. Das Manuskript I stellte zum ersten Mal die Expression von CXCR4 in thalamokortikalen Fasern dar und dass deren effizientes Wachstum abhängig von der CXCL12/CXCR4-Interaktion ist. Auch wenn die eigentliche Thalamus-Bildung weitgehend ohne Beteiligung von CXCL12 abläuft, so wird die axonale Verknüpfung zwischen Thalamus und Kortex von den wachstumsfördernden Eigenschaften des Chemokins beeinflusst. Bei Verlust von CXCL12 kommt es zur intrakortikalen Verringerung des thalamokortikalen Axon (TCA)-Wachstums, wohingegen Überexpression von CXCL12 die Entwicklung bis hin zur frühreifen CP-Invasion beschleunigt. Das thalamische Axon-Wachstum scheint dabei zum Teil von der Transkriptions-Dynamik und der Überlagerung der Signalwege von CXCL12/CXCR4 und Slit1/Robo1 kontrolliert zu werden. In einer frühen Phase, die mit schnellem Axon-Voranschreiten einhergeht, führt die hohe CXCL12/CXCR4-Interaktion und eine relativ niedrige Expression von Slit1/Robo1 zu Wachstumsförderung. Danach werden die Axone, vermittelt über verstärkte Slit1/Robo1- und verringerte thalamische CXCR4-Expression, verlangsamt (Mire et al. 2012). Zusätzlich verringert Slit1 die wachstumsfördernden Eigenschaften der restlichen CXCL12/CXCR4-Signaltransduktion. Einen entscheidenden Beitrag zur Expansion der thalamischen Fasern über den Kortex leistet CXCL12, das in der SVZ von Projektionsneuron-Vorläufern (IPC) gebildet wird. Es verknüpft dabei kortikale Neurogenese mit der effizienten Versorgung zukünftiger Netzwerk-Pendants, welche in unserer Analyse neben thalamokortikalen Fasern auch inhibitorische GABAerge Interneurone beinhaltete.

Die Migration zerebrokortikaler Interneurone und GnRH-Neurone hängt deutlich von dem Zusammenspiel des CXCL12/CXCR4/CXCR7-Systems ab. Die chemotaktische Wanderung dieser Nervenzellen wird hauptsächlich über die CXCL12/CXCR4-Interaktion vermittelt, da das Fehlen eines der beiden Gene (*Cxcl12*$^{-/-}$ und *Cxcr4*$^{-/-}$) bzw. des CXCR4-Rezeptorproteins eine fehlerhafte Verteilung zur Folge hat. Interessanterweise zeigen CXCR7-defiziente Mutanten meist einen ähnlichen Migrations-Phänotyp. Die Manuskripte II und III erklären dies hauptsächlich durch die CXCR7-*scavenger*-Funktion. Dabei bindet CXCR7 seinen Liganden CXCL12 mit hoher Affinität, internalisiert ihn, führt ihn dem Abbau zu und kehrt an die Plasmamembran zurück, wo dieser Kreislauf neu beginnen kann (Boldajipour et al.

2008, Hoffmann et al. 2012, Naumann et al. 2010). Über fluoreszenzmarkiertes CXCL12 (transgene CXCL12-RFP-Maus und synthetisiertes CXCL12-Atto565) wurde im Manuskript II erstmals die Aufnahme und Akkumulation von CXCL12 über CXCR7 in migrierenden kortikalen Interneuronen im Säugetier dargestellt. Zusätzlich zeigte diese Technik, dass CXCR7 die lokale Verteilung von CXCL12 im zerebralen Kortex und nasalen Mesenchym maßgeblich beeinflusst. Durch diese modulatorischen Eigenschaften titriert CXCR7 die CXCL12-Spiegel und schützt dadurch CXCR4 vor CXCL12-vermittelter Hyperaktivierung und dem daraus resultierenden verstärkten Abbau. Dabei kann diese CXCR7-Modulation entweder zellautonom oder nicht-zellautonom ablaufen.

5.1 Der atypische Chemokin-Rezeptor CXCR7 funktioniert zellautonom und nicht-zellautonom

GABAerge kortikale Interneurone sind während ihrer Migration im zerebralen Kortex eine der wenigen Zellpopulationen des Organismus, welche beide CXCL12-Rezeptoren CXCR4 und CXCR7 tragen (Sanchez-Alcaniz et al. 2011, Wang et al. 2011). In der vorgelegten Arbeit und in zuvor veröffentlichten Publikationen konnte gezeigt werden, dass CXCR7 zellautonom und nicht-zellautonom Einfluss auf die tangentiale Interneuron-Migration im Kortex nimmt (Sanchez-Alcaniz et al. 2011, Wang et al. 2011). Seine nicht-zellautonome Funktion übt CXCR7 durch eine frühe CP-Expression (~E13) aus. Nimmt man diese frühe *Cxcr7*-Expression konditional aus der Projektionsneuron-Linie (*Emx1*-Cre-Maus) heraus, so kommt es zu intrakortikaler Fehlverteilung von Interneuronen (Wang et al. 2011). Es wurde diskutiert, dass CXCR7 in der CP über die *scavenger*-Funktion einen CXCL12-Gradienten erzeugt und dadurch das Wanderungsverhalten indirekt beeinflusst. Darüber hinaus konnten zwei direkte, zellautonome Aufgaben von CXCR7 in migrierenden Interneuronen identifiziert werden. Zum einen führt die CXCL12-Bindung an CXCR7 vermittelt über β-Arrestin zur Aktivierung der MAP-Kinase (Wang et al. 2011). Zum anderen titriert CXCR7 über seine *scavenger*-Funktion die lokale Menge von CXCL12 und schützt dadurch CXCR4 vor Überaktivierung durch CXCL12 und dem damit einhergehenden verstärkten Abbau (Manuskript II, Sanchez-Alcaniz et al. 2011). Fehlt oder blockiert man CXCR7, so wird CXCL12 nicht mehr effizient internalisiert und reichert sich im Kortex an. Dadurch entstehen erhöhte CXCL12-Spiegel, die nun CXCR4 gesteigert aktivieren, internalisieren und dem Abbau zuführen. Über diesen Prozess wird die CXCR4-Proteinmenge deutlich reduziert, was wiederrum zu einer starken Abnahme der CXCL12/CXCR4-Interaktion und der chemotaktischen Migration in MZ und SVZ führt. Ohne CXCR7 kommt es also indirekt zu

einer funktionellen Reduktion bzw. zum Verlust der CXCR4-vermittelnden Signalkaskade, was die Ähnlichkeit der interneuronalen Migrationsdefekte in den *Cxcl12*$^{-/-}$, *Cxcr4*$^{-/-}$ und *Cxcr7*$^{-/-}$ Hirnen erklären kann (siehe hierzu Manuskript II). Der Vergleich zwischen *Cxcl12*$^{-/-}$, *Cxcr7*$^{-/-}$ und *Cxcl12*$^{-/-}$;*Cxcr7*$^{-/-}$ Embryonen deutete außerdem darauf hin, dass kortikales CXCL12 der einzige endogene Ligand für CXCR4 ist und dass der Verlust von CXCR4 im *Cxcr7*$^{-/-}$ Kortex durch CXCL12 herbeigeführt wird, da es durch zusätzliches Ausschalten von CXCL12 (*Cxcl12*$^{-/-}$;*Cxcr7*$^{-/-}$ Doppel-Mutante) zur Rettung des CXCR4-Proteins kommt. Allerdings kommt es auch in *Cxcl12*$^{-/-}$;*Cxcr7*$^{-/-}$ Embryonen zu einer Fehlverteilung der Interneurone im zerebralen Kortex, was wahrscheinlich auf das Fehlen des CXCL12/CXCR4-Signalwegs zurückzuführen ist. Eine mögliche Erklärung kann die fehlende Aktivierung der trimeren G-Proteine und ihrer inhibitorischen α-Untereinheit ($G_{\alpha i}$) liefern. So resultiert eine interneuron-selektive Blockade dieser G-Proteine über Pertussis-Toxin in einem Interneuron-Migrations-Phänotyp, der mit oben genannter Fehlverteilung vergleichbar ist (Wang et al. 2011).

Neben der „Feintitration“ von CXCL12 für eine homöostatische CXCR4-Funktion kann CXCR7 zellautonom bzw. nicht-zellautonom einen Chemokin-Gradienten hervorbringen und dadurch die gerichtete Chemotaxis modulieren. Dies trifft, wie oben beschrieben, auf die zerebrokortikalen Interneurone der Säugetiere zu und wird durch Arbeiten am Zebrafisch unterstützt (Boldajipour et al. 2008, Dona et al. 2013). Hier wandern primordiale Keimzellen (PGC) und Vorläuferzellen des lateralen Seitenlinienorgans geführt über CXCL12, entlang der rostro-kaudalen Achse (David et al. 2002, Doitsidou et al. 2002). Die PGC orientieren sich an einem CXCL12-Gradienten, der durch das umliegende somatische Gewebe über CXCR7 gebildet wird (nicht-zellautonom) (Boldajipour et al. 2008). Die Vorläuferzellen des lateralen Seitenlinienorgans generieren den CXCL12-Gradienten selbst (zellautonom), wobei CXCR7 und CXCR4 an diesem Prozess beteiligt sind (Dona et al. 2013). Dabei wird ein „CXCR4/CXCL12-signalgebender Gradient“ zwischen vorderem und hinterem Ende der migrierenden Vorläuferpopulation durch CXCR4-Rezeptor-Umsatz bestimmt. Zusätzlich wird im hinteren Ende CXCR7, der über seine *scavenger*-Funktion einen CXCL12-Gradienten ermöglicht, exprimiert. In *cxcr7*$^{-/-}$ Fischen kommt es zu einer fehlerhaften Lokalisation der Vorläuferzellen, was durch genetisches Einbringen einer externen, nicht-zellautonomen Quelle (posterioren Seitenlinienorgan-Nerv) von CXCR7 gerettet werden kann. Im Manuskript III wurde bei der GnRH-Neuron-Migration durch das nasale Mesenchym im Mausmodell ebenfalls ein nicht-zellautonomer CXCR7-*scavenger*-Effekt nachgewiesen. Hier beeinflussen CXCR7-exprimierende nasale Mesenchymzellen die lokale Verfügbarkeit von

CXCL12. Ohne CXCR7 kommt es zur gesteigerten CXCL12-vermittelten CXCR4-Internalisierung und –Proteinabnahme, was die chemotaktische Migration der GnRH-Neurone in Richtung Vorderhirn stört. Zusätzlich wurde eine Umverteilung von CXCL12 im nasalen Kompartiment und eine ektope CXCL12-Akkumulation in CXCR4-exprimierenden Zellen (auch hier ist CXCR4 reduziert) beobachtet. Es ist vorstellbar, dass CXCR7, welcher in einem rostro-kaudalen bzw. ventro-dorsalen Gradienten exprimiert ist, einen CXCL12-Gradienten im nasalen Mesenchym erzeugen kann und dadurch die gerichtete GnRH-Neuron-Migration gewährleistet.

Zusammenfassend lässt sich schlussfolgern, dass CXCR7 zellautonom und nicht-zellautonom agiert und dass er dabei, begründet durch zahlreiche *in vivo* Befunde, seine Hauptfunktion als CXCL12-*scavenger* ausübt.

5.2 CXCL12 ist an der frühen Ausbildung zerebrokortikaler Netzwerke beteiligt

Die komplexe Struktur des zerebralen Kortex wird während ihrer Entstehung aus lokalen und exogenen Zellen bzw. deren Ausläufern zusammengesetzt. Kortikale exzitatorische Projektionsneurone entstehen lokal durch Zellteilung aus radialen Gliazellen oder basalen Vorläuferzellen (IPC), wandern radial und bilden in einem „*inside-out*“ Muster die sechs Schichten des zerebralen Kortex (Abb. 2) (Marin und Muller 2014). Das evolutionäre Aufkommen der basalen Vorläuferzellen wird u. a. für die Expansion des Säugetier-Kortex verantwortlich gemacht (Martinez-Cerdeno et al. 2006, Vasistha et al. 2014). Da diese Expansion kortikaler Projektionsneurone nach einer erhöhten Anzahl externer Eingänge und mehr inhibitorischer Regulation „verlangt“, mussten sich Mechanismen evolutionär entwickeln, die diese neuen Anforderungen bewerkstelligen konnten. Zur Zeit ist wenig über diese Mechanismen, welche lokale, kortikale Neurogenese und Rekrutierung exogener Komponenten koordinieren, bekannt. Das Manuskript I zeigt erstmals, dass CXCL12 ein mögliches Molekül für die frühe Koordination dieser Prozesse ist. Eine einzigartige Konstellation zeigt sich dabei in der SVZ/IZ: Hier wandern und teilen sich kortikale Projektionsneuron-Vorläufer und exprimieren gleichzeitig *Cxcl12* (Tiveron et al. 2006), was wiederrum für die effiziente Verteilung kortikaler Interneurone und das Einwachsen von thalamischer Axone in den Kortex sorgt.

Im Vorfeld der eigenen Arbeit wurde gezeigt, dass die Transkriptionsfaktoren Pax6 und Tbr2 essentiell für die Zellteilung und die *Cxcl12*-Expression sind (Sessa et al. 2010, Tiveron et al. 2006). Der Verlust dieser Transkriptionsfaktoren führt zu Entwicklungsdefekten im Kortex – z. B. zu Migrationsdefekten der Interneurone. Da der Verlust der Faktoren jedoch eine

Vielzahl unbekannter Auswirkungen nach sich ziehen kann, können diese Defekte nicht zweifelsfrei auf das Fehlen von CXCL12 zurückgeführt werden. Deshalb wurde *Cxcl12* gezielt über eine *Tbr2*-Cre in den Vorläufern der Pyramidenzellen deletiert. Hierdurch sollte überprüft werden, ob CXCL12 die Bildung kortikaler Pyramidenzellen und das Einwandern von Interneuronen verknüpft. Tatsächlich war die Interneuron-Migration in den konditionalen *Cxcl12*-Mutanten gestört. Es kam zur Interneuron-Reduktion entlang der latero-medialen SVZ-Achse und zur Verlagerung der Interneurone in die oberen Schichten. Letzteres lässt sich vermutlich durch die bestehende CXCL12-Expression in den Meningen erklären. Darüber hinaus wurde zum ersten Mal gezeigt, dass eine zweite exogene kortikale Komponente – die thalamokortikalen Axone – abhängig von CXCL12 aus kortikalen Vorläuferzellen ist.

Zur Zeit ist CXCL12 das einzig bekannte Molekül, welches die tangentiale Interneuron-Migration im zerebralen Kortex vermittelt (Marin 2013). Folglich ist es nach den hier präsentierten Ergebnissen auch der erste und einzig bekannte Ligand, welcher sowohl die tangentiale Migration als auch das Wachstum thalamokortikaler Axone direkt beeinflusst. Ob der CXCL12-vermittelte Effekt in Interneuronen und den thalamischen Axonen ähnlich funktioniert, ist noch nicht geklärt. Prinzipiell handelt es sich um unterschiedliche Prozesse, da bei der Wegfindung der Interneurone die ganze Zelle wandert, wohingegen das thalamokortikale Axon über den Wachstumskegel navigiert (Marin et al. 2010, O'Donnell et al. 2009). CXCL12 scheint dabei auch unterschiedlich auf diese Prozesse zu wirken. In Interneuronen vermittelte es die klassische Chemotaxis, wobei es in thalamokortikalen Axonen eher wachstumsfördernd wirkt, da nur schnelleres Wachstum, aber kein offensichtliches Fehlleiten beobachtet wurde. Eine Wachstumsförderung kann hierbei direkt über CXCL12 (Manuskript I, *in vitro* Daten) oder indirekt über eine CXCL12-vermittelte Steigerung der Gewebspermissivität, was beispielsweise durch Reduktion repulsiver Eigenschaften von anderen Molekülen realisiert werden kann, erfolgen. In der Tat zeigen einige *in vitro* Befunde, dass CXCL12 die repulsive Wirkung von Slit2 auf retinale Ganglienzell-Axone und Sema3A auf Hinterwurzel-Ganglien-Axone mindern kann (Chalasani et al. 2003, Chalasani et al. 2007, Twery und Raper 2011). Es ist denkbar, dass CXCL12 aus kortikalen Vorläuferzellen ebenfalls den repulsiven Einfluss von Sema3A auf thalamische Axone moduliert, da sie überlappend in der SVZ/IZ gebildet werden (Ruediger et al. 2013). Aus *in vitro* und *in vivo* Experimenten wurde eine dynamische Interaktion zwischen dem CXCL12/CXCR4 und Slit1/Robo1 System abgeleitet (Manuskript I). Die Wachstumsgeschwindigkeit der thalamokortikalen Fasern wird danach u. a. von einer

inversen Transkription der beiden Systeme gesteuert. Anfänglich ist die „bremsende“ Slit1/Robo1-Expression niedrig und die CXCR4-Expression hoch, was zu schnellem intrakortikalen Wachstum, vermittelt über CXCL12, führt (Manuskript I, Mire et al. 2012). In *Cxcl12*$^{-/-}$ Embryonen war eine verfrühte Hochregulation von *Robo1* in lateralen thalamischen Kernen beobachtbar, was zusätzlich zur fehlenden CXCL12/CXCR4-Interaktion die thalamischen Axone in diesem Genotyp verlangsamte. In Kontroll-Embryonen kommt es erst in einer späteren Phase zur Verlangsamung der thalamokortikalen Fasern, welches durch die Abnahme der thalamischen CXCR4-Expression und erhöhter Slit1/Robo1-Expression erklärbar ist. Des Weiteren zeigten *in vitro* Untersuchungen, dass Slit1 die wachstumsfördernden Eigenschaften von CXCL12 reduzieren kann. Slit1 wird in der CP oberhalb der einwachsenden Axone gebildet und könnte somit zum Übergang von schnellem auf langsames Wachstum beitragen. Dies wird durch Studien an *Slit1*$^{-/-}$ und *Robo1*$^{-/-}$ Embryonen unterstützt, da es hier zu einer schnelleren intrakortikalen Ausbreitung thalamischer Axone kommt (Andrews et al. 2006, Mire et al. 2012). Auch das CXCL12-Überexpressions-Mausmodell besitzt einen ähnlichen Phänotyp, was zusätzlich eine Interaktion der beiden Signalwege vermuten lässt.

Bei der Migration von Interneuronen innerhalb des Kortex scheinen diese Signalwege unabhängig voneinander zu funktionieren. Während die Migration vom *Pallium* zum *Subpallium* Slit/Robo-abhängig und CXCL12/CXCR4-unabhängig zu sein scheint, ist das Ganze für die tangentiale Migration im Kortex umgekehrt (Marin 2013). So zeigen beispielsweise *Slit1*$^{-/-}$;*Slit2*$^{-/-}$ Doppelmutanten keine offensichtliche Störung der tangentialen Interneuron-Migration im Kortex (Marin et al. 2003).

Das teilweise unterschiedliche Ansprechen der Interneurone und thalamischen Axone auf die kortikalen „Lenkmoleküle“ lässt sich wahrscheinlich durch unterschiedliche Transkription von Rezeptoren und dem Aktivieren verschiedener Signalwege erklären. So soll die Slit/Robo-Interaktion beispielsweise das Axon-Wachstum durch Phosphorylierung der fokalen Adhäsions-Kinase (*focal adhesion kinase*, FAK) modulieren (Navarro und Rico 2014). Im Gegensatz dazu bewirkt der konditionale *knockout* der *FAK* in kortikalen Interneuronen keine Migrationsdefekte (Valiente et al. 2011). Dies ist nicht intuitiv, da die FAK ein wichtiger Vermittler der Rezeptor/Zytoskelett-Interaktion ist und die zelluläre Maschinerie im interneuronalen Leitfortsatz und axonalem Wachstumskegel ähnlich funktioniert (Dent und Gertler 2003, Peyre et al. 2015). Dabei spielen dynamische Ab- und Aufbau-Prozesse von F-Aktin und Mikrotubli eine entscheidende Rolle. Kürzlich wurde CXCL12 mit der Spaltung bestimmter Aktin-Verzweigungen und Stabilisierung von

Mikrotubuli in Interneuronen in Verbindung gebracht (Lysko et al. 2014). Unter CXCL12/CXCR4-Interaktion kommt es durch Aktivierung des G_i-Proteins zu einer Abnahme des cAMP-Spiegels und zu einer Aufhebung der Repression von Calpain und Doublecortin (DCX). Calpain spaltet nun Cortactin, was zur Aktin-Konsolidierung und zu zeitlich verkürzter Leitfortsatzverzweigung führt. Die DCX-Affinität zu Mikrotubuli steigt (durch reduzierte Phosphorylierung), wodurch es die Mikrotubuli-Bündelung unterstützt. Zusammengenommen kommt es durch weniger Verzweigungs(*branching*)-Ereignisse zu einer erhöhten Interneuron-Geschwindigkeit. Es ist vorstellbar, dass die CXCL12/CXCR4-Interaktion über ähnliche Mechanismen die Wachstumsgeschwindigkeit von thalamokortikalen Fasern erhöht.

Die geschilderten Beobachtungen und Schlussfolgerungen aus Manuskript I beziehen sich alle auf embryonale, neuronale Entwicklungsprozesse. Es ist nicht auszuschließen, dass es sich um transiente Ereignisse handelt und dass die Phänotypen (TCA-Wachstumsverlangsamung und Interneuron-Migrationsphänotyp in *Cxcl12*-Mutanten) während der nachfolgenden Entwicklung kompensiert werden. Da die Verknüpfung zwischen CXCL12 und dem Wachstum thalamischer Axone durch das Manuskript I erstmals beschrieben wurde, bedarf es weiterer Untersuchungen, um Rückschlüsse auf das weitere Entwicklungsgeschehen zu ziehen. Es könnte sein, dass die „zurückliegenden“ thalamischen Axone in den *Cxcl12*-Mutanten während der „Warteperiode“ aufholen (Lopez-Bendito und Molnar 2003) und das Einwachsen in die kortikalen Schichten CXCL12-unabhängig geschieht. Auf diese Weise würde ein früher Entwicklungsdefekt kompensiert werden. Eine umgekehrte Hypothese würde man formulieren, wenn die verlangsamten thalamokortikalen Fasern sich nicht normal über den Kortex ausbreiten und ektope Verknüpfungen mit anderen kortikalen Arealen bzw. Schichten herstellen. Eine ähnliche Beobachtung wurde für die Migration kortikaler Interneurone, bei denen CXCR4 selektiv entfernt wurde, festgestellt (Li et al. 2008, Tanaka et al. 2010). Hierbei konnte gezeigt werden, dass CXCR4 für die Verteilung über den Kortex in latero-medialer bzw. rostro-kaudaler Richtung und in den kortikalen Schichten entscheidend ist. In kortikalen Regionen mit reduzierter Interneuron-Dichte kommt es zusätzlich zur Störung der inhibitorischen Signale (Li et al. 2008). Weiterführende Einblicke in die CXCL12-vermittelte Verteilung kortikaler Interneurone könnte der *Tbr2*-Cre-vermittelte konditionale *knockout* von *Cxcl12* liefern. Mit postnatalen Analysen könnte man die besondere Rolle von neuronal-gebildetem CXCL12 auf diesen Prozess entschlüsseln.

5.3 Das CXCL12/CXCR4/CXCR7-System verbindet Grundlagenforschung mit aktueller medizinischer Forschung

Die hier vorgelegte Dissertation beschäftigt sich mit der Entstehung neuronaler Strukturen unter dem Einfluss des Chemokins CXCL12 und seiner Rezeptoren CXCR4 und CXCR7. Dabei vereinen die Manuskripte I – III entwicklungs-neurobiologische und pharmakologische Fragestellungen. Weiterführend geben sie Aufschluss über die *in vivo* Liganden/Rezeptor-Interaktion und können dadurch Informationen für die aktuelle medizinische Forschung liefern.

Die Verbindung zwischen CXCL12 und Entwicklungsdefekten, die zu Krankheiten führen können, hat in den letzten Jahren an Interesse gewonnen. Kortikale GABAerge Interneurone werden eng mit der Entstehung psychischer Störungen, wie Schizophrenie und Autismus, in Verbindung gebracht (Benes 2000, Blatt und Fatemi 2011, Lewis et al. 2005). So kommt es beispielsweise im Mausmodell des Deletionssyndroms 22q11, das beim Menschen eng mit psychiatrischen Störungen assoziiert wird, zu einer veränderten *Cxcl12*/*Cxcr4*-Expression und zu einer Fehlverteilung von Interneuronen (Meechan et al. 2012, Toritsuka et al. 2013). Das Kallmann-Syndrom wird als eine weitere Krankheit, an deren Entstehung CXCL12 beteiligt ist, angesehen (Schwarting et al. 2007). Bei diesem Syndrom kommt es zu einer fehlerhaften Migration von GnRH-Neuronen und dadurch zu hypogonadotropem Hypogonadismus bis hin zum Verlust des Geruchssinns (Dode und Hardelin 2004, Quinton et al. 1997).

Das Manuskript I zeigt, dass die CXCL12/CXCR4-Interaktion für ein effizientes intrakortikales Wachstum von thalamokortikalen Axonen relevant ist. Dieser Befund könnte für die therapeutische Forschung der Axon-Regeneration von Interesse sein. Hier konnte bereits gezeigt werden, dass beschädigte Axone im Rückenmark und im optischen Nerv unter Entzündungsstimulus oder CXCL12 regeneratives Verhalten zeigen (Heskamp et al. 2013, Jaerve et al. 2012). Dabei wirkt CXCL12 auf Neurit-Auswachsen, Proliferation, Überleben, Differenzierung von endogenen Zellen und auf die Rekrutierung transplantierter Stammzellen bzw. Vorläuferzellen zum Verletzungsort.

CXCR4 hat sich als ein interessantes Ziel für die Stammzell- bzw. Krebsforschung herausgestellt. So wurden zahlreiche CXCR4-Inhibitoren aus den molekularen Gruppen der Antikörper, Chemokin-Analoga, endogenen Defensinen, Derivate des Pfeilschwanzkrebs-proteins Polyphemusin und virusabstammenden Chemokin-Bindeproteinen sowie nieder-molekularen Antagonisten entwickelt (Bachelerie et al. 2014). Darunter befinden sich einige Substanzen, die bereits in klinischen Studien getestet werden (Burixafor, POL6326, MDX-1338, BKT140) und der zugelassene Antagonist AMD3100 (Plerixafor oder Mozobil; Sanofi,

Paris, Frankreich). AMD3100 und Granulozyten-Makrophagen-Kolonie-stimulierender Faktor (GM-CSF) werden zur Freisetzung von Stammzellen bei Knochenmarktransplantationen für Patienten mit Multiplem Mylom und Non-Hodgkin-Lymphom eingesetzt. Dabei bindet AMD3100 reversibel an CXCR4, wodurch die CXCL12-Bindung geblockt wird und die Stammzellen das Knochenmark verlassen. Das WHIM Syndrom, das eine durch Warzen, Hypogammaglobulinämie und *Myelokathexis* gekennzeichnete Immunschwächekrankheit ist, könnte eine weitere Anwendung von AMD3100 darstellen (Bachelerie et al. 2014). Dieses Syndrom wird auf einen verkürzten C-Terminus des CXCR4 zurückgeführt. Dieser beinhaltet regulatorische Phosphorylierungsstellen, die für Desensitisierung und Internalisierung des Rezeptors relevant sind (Busillo und Benovic 2007, Mueller et al. 2013). Es kommt unter Agonist-Bindung an dem WHIM-CXCR4 zu einem *gain of function* in der Signaltransduktion (G-Protein-Kopplung und β-Arrestin-Signal sind erhöht). Durch geringe Dosen von AMD3100 versucht man nun, diesen hyperaktiven CXCR4 zu dämpfen und die WHIM Symptome zu mildern (Bachelerie et al. 2014).

Das wachsende Wissen über CXCR7 ermöglicht es, weitere Strategien für die Modulation des CXCL12/CXCR4/CXCR7-Systems unter pathophysiologischen Bedingungen zu entwickeln. So untersucht die Firma Chemocentryx (Kalifornien, USA) beispielsweise nieder-molekulare Bindungspartner am CXCR7. Die Substanz CCX650 befindet sich in der vorklinischen Testphase für die Therapie von *Glioblastoma Multiforme*. Die verwandten Moleküle CCX771 und CCX733 konkurrieren bereits in niedriger Dosis (~5 nM) mit CXCL12 um die Bindung an CXCR7. Dabei wirken sie mit Hinblick auf die β-Arrestin-Rekrutierung als Agonist (Luker et al. 2010, Zabel et al. 2009). In der Arbeitsgruppe um Prof. Stumm konnten wir ebenfalls zeigen, dass Antagonisten und Agonisten an CXCR4 und CXCR7 wirksam sind. Mittels *in vitro* und *in vivo* Studien wurde dargelegt, dass AMD3100 CXCR4 blockiert und ihn in einem inaktiven/nicht-phosphorylierten Zustand an der Zellmembran hält (Manuskript II, Mueller et al. 2013, Schultheiss et al. 2013). Zusätzlich antagonisiert AMD3100 ein Überangebot von CXCL12 und schützt dadurch CXCR4 vor Hyperaktivierung und dem damit einhergehenden gesteigerten Abbau (Manuskript II). Im Organismus bietet CXCR7 über seine *scavenger*-Aktivität eine ähnliche Funktion. Er steuert die lokale Verfügbarkeit bzw. den Spiegel von CXCL12 und schützt dadurch CXCR4 vor übersteigerter Aktivierung und Herunterregulation. Dies wurde neben den beschriebenen *knockout*-Mauslinien auch durch eine CXCR7-Blockade mit CCX771 belegt (Manuskript II). Kortikale Vorläuferzellen konnten hier CXCL12 unter

CXCR7-Blockade nicht mehr effektiv internalisieren und akkumulieren, was dann durch erhöhte CXCL12-Spiegel zum Abbau von CXCR4 führte.

Ein weiteres großes CXCL12-assoziiertes medizinisches Forschungsfeld ist die Krebsforschung. CXCR4 und CXCR7 wurden in verschiedenen epithelialen, mesenchymalen und hämatopoetischen Krebserkrankungen nachgewiesen und wurden mit Proliferation, Tumorwachstum, Angiogenese und Metastasierung assoziiert (Balkwill 2004, Freitas et al. 2014). Letztere ist besonders interessant, da CXCR4-positive Tumore nach *Intravasation* und Zirkulation im Blutstrom besonders CXCL12-reiche Organe, wie Knochenmark, Lunge, Leber, Hirn und Lymphknoten befallen (Hattermann und Mentlein 2013). Diese Eigenschaften machen das CXCL12/CXCR4/CXCR7-System zu einem wichtigen Ziel für die Entwicklung neuer medizinischer Strategien gegen Krebs.

6 Schlussfolgerung

In einem komplexen Zusammenspiel regulieren CXCL12 und seine Rezeptoren CXCR4 und CXCR7 die Entstehung neuronaler Strukturen. Bei diesem Prozess wird CXCL12 von neuronalen und nicht-neuronalen Zellen gebildet. Nicht-neuronales CXCL12 im Nasenmesenchym leitet GnRH-Neurone in Richtung Vorderhirn. Zerebrokortikale Interneurone folgen stereotypen Routen entlang der CXCL12-Expression. Dabei hält meningeales/nicht-neuronales CXCL12 die Interneurone in der MZ und neuronales CXCL12, welches von Projektionsneuron-Vorläufern (IPC) gebildet wird, in der SVZ. Letzteres reguliert zusätzlich das intrakortikale Wachstumsverhalten von thalamokortikalen Axonen. Daraus ergibt sich eine einzigartige Konstellation, bei der kortikale Vorläuferzellen bzw. neugeborene Neurone CXCL12 exprimieren, um die effiziente Versorgung mit ihren späteren Netzwerkpartnern aus exogener Herkunft zu gewährleisten. In den vorliegenden Experimenten wurde dies nur für die pränatale Entwicklung nachgewiesen. Es wäre in nachfolgenden Studien interessant, welchen Einfluss neuronales CXCL12 für die weitere Entwicklung bzw. Entstehung anderer neuronaler Strukturen hat.

Die chemotaktische bzw. wachstumsfördernde Eigenschaft von CXCL12 wird hauptsächlich über CXCR4 vermittelt. Dabei hängt sowohl die Rezeptoraktivierung wie auch Rezeptormenge von der Anwesenheit des Liganden ab. So führen hohe CXCL12-Mengen bei kortikalen Interneuronen und GnRH-Neuronen zur Hyperaktivierung von CXCR4 und zu einem verstärkten Abbau. Vor diesem Prozess kann CXCR7 mittels seiner CXCL12-*scavenger*-Fähigkeit schützen. Hierbei wird CXCL12 von CXCR7 mit hoher Affinität gebunden, internalisiert und dem Abbau zugeführt, wodurch seine lokale Verfügbarkeit reguliert wird. Zusätzlich wurde gezeigt, dass CXCR7 über β-Arrestin die MAP-Kinase-Signalkaskade moduliert. Weiterführend könnte man prüfen, welche neuronalen Strukturen unter dem Einfluss der CXCR7-*scavenger*-Funktion und/oder β-Arrestin-Signaltransduktion entstehen. *In vivo* Studien mit internalisierungsdefizienten bzw. β-Arrestin-rekrutierungsunfähigen CXCR7-Mutanten würden die Interaktion mit CXCL12 weiter konkretisieren. Dies ist vor allem für Gewebe bzw. Tumorarten von Interesse, die ausschließlich CXCR7 exprimieren und bei denen der CXCL12-*scavenger*-Effekt eine untergeordnete oder keine Rolle spielt.

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8 Anhang

8.1 Ehrenwörtliche Erklärung

Hiermit erkläre ich, dass mir die Promotionsordnung der Medizinischen Fakultät der Friedrich- Schiller-Universität bekannt ist,

ich die Dissertation selbst angefertigt habe und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in meiner Arbeit angegeben sind,

mich folgende Personen bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts unterstützt haben: Prof. Dr. Ralf Stumm (Betreuer), Dr. Dagmar Schütz, Dr. Andrea Kliewer, Arite und Maria Schrehardt,

die Hilfe eines Promotionsberaters nicht in Anspruch genommen wurde und dass Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen,

dass ich die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht habe und

dass ich die gleiche, eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung nicht bei einer anderen Hochschule als Dissertation eingereicht habe.

Jena, 24.06.2015

Ort, Datum Unterschrift P.Abe

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8.3 Wissenschaftlicher Beitrag

Publikationen

Abe, P., Mueller, W., Schutz, D., Mackay, F., Thelen, M., Zhang, P. and Stumm, R. (2014). CXCR7 prevents excessive CXCL12-mediated downregulation of CXCR4 in migrating cortical interneurons. *Development*. (impact factor 2013: 6.273)

Abe, P., Schultheiss, C. , Hoffmann, F., Mueller, W., Kreuder, A. E., Schutz, D., Haege, S., Redecker, C., Keiner, S., Kannan, S. et al. (2013). CXCR4 prevents dispersion of granule neuron precursors in the adult dentate gyrus. *Hippocampus* **23**, 1345-1358. (impact factor 2012: 5.492)

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Poster

07/2012 FENS Barcelona, Spanien. Defective interneuron migration in the CXCR7-deficient cortex is due to exaggerated homologous CXCR4 regulation

12/2013 FENS winter school Obergurgl, Österreich. The chemokine CXCL12 influences thalamic development

05/2014 Cortical Development Chania, Griechenland. CXCR7 prevents excessive CXCL12-mediated down-regulation of CXCR4 in migrating cortical interneurons

03/2015 Konferenz der Neurowissenschaftlichen Gesellschaft, Göttingen, Deutschland. Cortical progenitors release the chemokine CXCL12 (SDF-1) to promote ingrowth of thalamocortical afferents

05/2015 Adult Neurogenesis: Evolution, Regulation and Function, Dresden, Deutschland. CXCR4 Prevents Dispersion of Granule Neuron Precursors in the Adult Dentate Gyrus